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STUDIES ON THE ROLE OF AUTOANTIBODIES AND AUTOANTIGENS IN RHEUMATOID ARTHRITIS AND MYOSITIS

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Studies on the role of autoantibodies and autoantigens in rheumatoid arthritis and myositis

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"Para ser grande sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive"

Ricardo Reis, heterónimo de Fernando Pessoa

ABSTRACT

A major population of patients affected with rheumatoid arthritis (RA) and inflammatory idiopathic myopathies (IIM, collectively called myositis) is characterized by the presence of autoantibodies. The pathogenic impact of anti-citrullinated protein/peptide (ACPA) or anti-histidyl transfer RNA synthetase (HisRS; Jo1) autoantibodies remains largely unknown. The aims of the thesis were to understand the molecular mechanisms underlying the autoimmune component (specifically, the auto-antibody and -antigen dynamics) in RA and myositis; and to develop ACPA neutralizing compounds.

Paper I: Purified anti-cyclic citrullinated peptide antibodies (aCCP2 IgG) were estimated to represent ~2% of the total RA IgG pool, and demonstrated to display distinct and individual reactivities against citrullinated epitopes from fibrinogen, collagen type II, α-enolase and vimentin. *In-vivo* generated autoantigens in synovial tissue and fluid were recognized by aCCP2 IgG.

Paper II: Anti-CCP2 IgG purified according to the methodology described in paper I were injected in mice and shown to induce pain-like behaviour. The underlying molecular mechanism appears to be chemokine-dependent because 1) aCCP2 IgG promoted activation of murine osteoclasts with generation of CXCL1 (human IL-8); 2) aCCP2 IgG stained CD68-bearing macrophages/osteoclasts, closely localized to the calcitonin gene related peptide (CGRP)-positive sensory nerve fibers; 3) reparixin, a CXCL1/2 receptor antagonist, blocked aCCP2 IgG induced pain.

Papers III and IV: Endogenously citrullinated fibrinogen peptides were found to be recognized by a large portion of sera from the Epidemiological Investigation of RA (EIRA) cohort. CCP2+ RA sera displayed 65% and 15 % immune reactivity against peptides from the fibrinogen α chain Cit573 (563-583) and Cit591 (580-600), respectively. Peptides from the β chain, Cit72 (62-81) and Cit74 (62-81), were recognized by 35% and 53% of the CCP2+ RA patients. The same fibrinogen peptides were shown to *in vitro* individually bind and block purified aCCP2 IgG in a dose-dependent manner, displaying a maximum of 83% blockade. Approximately 90% autoantibodies were neutralized by Cit573 and Cit591 combined, and further improvement of the blocking capacity was registered (>90%) when incubating aCCP2 IgG with a cyclic version of Cit573.

Since the peptide Cit573 (also termed [Cit573]fib(563-583)) showed the best inhibition percentage, a truncated version was inserted into the stable framework sunflower trypsin inhibitor-1 (SFTI-1, and denoted s1[Cit573]fib(566-580)). This compound showed enhanced blocking capacity, 79% antibodies were neutralized with an estimated IC_{50} of 20 μM, in comparison to the linear counterparts (73% maximum inhibition with the $IC_{50:s}$ ranging from 59 to 123 μM). Stability in blood was also improved, with above 90% of s1[Cit573]fib(566-580) remaining after five hours, whereas the cyclic and linear counterparts were degraded after one hour. Using a mutant of s1, s2[Cit573,Arg575]fib(566-580), anti-[Cit573]fib(563-583) IgG (aCit573 IgG) were purified from RA plasma and estimated to comprise 0.33% of the total RA IgG pool. Binding affinity measurements demonstrated that aCit573 IgG bind

the mutant scaffold peptide s3[Cit573,Arg575]fib(566-580) with high affinity (K_d = 2 nM). Thus, subsets of ACPA recognizing citrullinated fibrinogen epitopes appear to be of high affinity.

Paper V: The cytoplasmic autoantigen HisRS was found in extracellular compartments (bronchoalveolar lavage, BAL, sera and plasma). High circulating levels of HisRS were found in myositis, and further increased in anti-HisRS+ patients; significant levels of HisRS were also detected in healthy individuals, whereas anti-HisRS IgG, IgA and IgM autoantibodies were only detected in sera and BAL from patients with myositis. In addition, anti-TRIM21 IgG were also identified in myositis BAL, positively correlating with the presence of anti-HisRS IgG. A so far uncharacterized factor in BAL of myositis patients was found to bind exogenous HisRS. In experiments addressing platelet activation, HisRS was found to trigger platelet activation in a dose-response dependent manner at low picomolar concentrations.

In conclusion, the development of a methodology to isolate autoantibodies from RA patients' plasma, serum and synovial fluid, provided opportunities to address the pathogenic role of ACPA. Purified aCCP2 IgG induced pain-like behaviour, raising a possible mechanism for the pain RA patients occasionally feel before clinical onset of disease or after being medicated and in remission. A stable, high affinity anti-citrullinated fibrinogen autoantibody blocking compound was designed and we propose ACPA neutralization with fibrinogen-derived peptides as a complimentary treatment strategy for ACPA+RA. ACPA pain mediated effect may also be a functional target, amenable for blocking. The presence of HisRS extracellularly suggests novel and so far undescribed functions, which merits further investigations. Finally, a possible coupled immune response among HisRS and TRIM21 in lungs of myositis patients provides new clues for the development of autoimmunity in myositis and the associated anti-synthetase syndrome.

LIST OF SCIENTIFIC PAPERS

I. **Affinity purified anti-citrullinated protein/peptide antibodies target antigens expressed in the rheumatoid joint**
E. Ossipova[*], **C. Fernandes-Cerqueira**[*], E. Reed, N. Kharlamova, L. Israelsson, R. Holmdahl, K.S. Nandakumar, M. Engström, U. Harre, G. Schett, A.I. Catrina, V. Malmström, Y. Sommarin, L. Klareskog, P-J. Jakobsson, and K. Lundberg
Arthritis Research & Therapy 2014, 16:R167

II. **Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism**
G. Wigerblad, D.B. Bas[*], **C. Fernandes-Cerqueira**[*], A. Krishnamurthy, K.S. Nandakumar, K. Rogoz, J. Kato, K. Sandor, J. Su, J.M. Jimenez -Andrade, A. Finn, A. Bersellini Farinotti, K. Amara, K. Lundberg, R. Holmdahl, P-J. Jakobsson, V. Malmström, A.I. Catrina, L. Klareskog, and C.I. Svensson
Ann Rheum Dis. 2015, Nov 27

III. **Targeting of anti-citrullinated protein/peptide antibodies in rheumatoid arthritis using peptides mimicking endogenously citrullinated fibrinogen antigens**
C. Fernandes-Cerqueira[*], E. Ossipova[*], S. Gunasekera, M. Hansson, L. Mathsson, A.I. Catrina, Y. Sommarin, L. Klareskog, K. Lundberg, J. Rönnelid, U. Göransson, and P-J. Jakobsson
Arthritis Research & Therapy 2015, 17:155

IV. **Design, Synthesis, Bioactivity Screening and Structural Characterization of Anti-Citrullinated Protein/Peptide Antibody Inhibitors**
S. Gunasekera, **C. Fernandes-Cerqueira**, S. Wennmalm, H. Wähämaa, Y. Sommarin, A.I. Catrina, P-J. Jakobsson, U. Göransson
Manuscript

V. **Characterization of extracellular histidyl-tRNA synthetase in myositis**
C. Fernandes-Cerqueira, A. Sohrabian, I. Albrecht, F. Mobarrez, A. Notarnicola, E. Ossipova, J. Lengqvist, M. Fathi, G.J. Pruijn, J. Grunewald, J. Rönnelid, I.E. Lundberg, and P-J. Jakobsson
Manuscript

[*]Equal contribution

ADDITIONAL PUBLICATIONS

Neutralization of anticitrullinated protein antibodies in rheumatoid arthritis - a way to go?
C. Fernandes-Cerqueira, L. Klareskog, and P-J. Jakobsson
Basic Clin Pharmacol Toxicol. 2014 Jan;114(1):13-7

IgG antibodies to cyclic citrullinated peptides exhibit profiles specific in terms of IgG subclasses, Fc-glycans and a fab-Peptide sequence
S.L. Lundström, **C. Fernandes-Cerqueira**, A.J. Ytterberg, E. Ossipova, A.H. Hensvold, P-J. Jakobsson, V. Malmström, A.I. Catrina, L. Klareskog, K. Lundberg, and RA. Zubarev
PLoS One. 2014 Nov 26;9(11)

Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone destruction
A. Krishnamurthy, V. Joshua, A.H. Hensvold, T. Jin, M. Sun, N. Pomiano-Vivar, A.J. Ytterberg, M. Engström, **C. Fernandes-Cerqueira**, K. Amara, M. Magnusson, G. Wigerblad, J. Kato, K.Tyson, S. Rapecki, K. Lundberg, S-B Catrina, P-J Jakobsson, C.I. Svensson, V. Malmström, L. Klareskog, H. Wähämaa, and A.I. Catrina
Ann Rheum Dis. 2015, Nov 26

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

aaRS	Aminoacyl-transfer (t) RNA synthetases
ACR	American College of Rheumatology
ACPA	Anti-citrullinated protein/peptide (auto)antibody(ies)
ADCC	Antibody dependent cellular cytotoxicity
Ala	Alanine
Anti-Jo1+/-;Anti-HisRS+/-	Anti-histidyl-tRNA synthetase (auto) antibody positive/negative
Anti-EJ	Anti-glycyl-tRNA synthetase antibody
Anti-Ha	Anti-tyrosyl-tRNA synthetase antibody
Anti-KS	Anti-asparaginyl-tRNA synthetase antibody
Anti-OJ	Anti-isoleucyl-tRNA synthetase antibody
Anti-PL-12	Anti-alanyl-tRNA synthetase antibody
Anti-PL-7	Anti-threonyl-tRNA synthetase antibody
Anti-Zo	Anti-phenylalanyl-tRNA synthetase antibody
APC	Antigen presenting cells
Arg	Arginine
Asp	Aspartic acid
ASS	Anti-synthetase syndrome
BAL	Bronchoalveolar lavage
BCR	B cell receptor
C	Antibody constant region
CCP	Cyclic citrullinated peptide
CCR5	C-C chemokine receptor type 5
CEP-1	Citrullinated α-enolase peptide 1 (amino acid residues 5-21)
Cit	Citrulline/Citrullinated
Cit/Arg573; [Cit/Arg573]fib(563-583)	Cit/Arg fibrinogen peptide (α chain amino acid residues 563-583)
Cit-fib	Cit fib peptide (β chain, amino acid residues 36-52)
Cit573Lin1; [Cit573]fib(566-580)	Cit fib peptide (α chain 566-580) linear 15 amino acid residues long
Cit573Lin2; [Cit573]fib(567-577)	Cit fib peptide (α chain 567-577) linear 11 amino acid residues long
Cit573Cyc; c[Cit573]fib(563-583)	Cyclic cit fib peptide (α chain amino acid residues 563-583)
Cit/Arg591	Cit/Arg fib peptide (α chain amino acid residues 580-600)
Cit/Arg72; Cit/Arg74	Cit/Arg fib peptide (β chain amino acid residues 62-81)
Cit-vim	Cit vimentin peptide (amino acid amino acid residues 60-75)
c[Cit/Arg/Ala573]fib(566-580)	Cyclic Cit/Arg/Ala fib peptide (α chain 563-583) 15 amino acid residues long
CD62P	P-selectin
CGRP	Calcitonin gene related peptide
CII; Cit-C1	Collagen type II; Cit triple helical peptide on CII (amino acid residues 359-369)
CTD	Connective tissue disorders
CXCL	Chemokine CXC motif ligand
DC	Dendritic cells
DM	Dermatomyositis
DMARD	Disease-modifying anti-rheumatic drug
DRG	Dorsal root ganglia
GC	Germinal centers
GluC	Glucocorticoids
GrB	Granzyme B
EIRA	Epidemiological Investigation of Rheumatoid Arthritis
ELISA	Enzyme-linked immunosorbent assay
EULAR	European League Against Rheumatism
Fab	Fragment antigen-binding
Fc	Fragment crystallizable region
FT	Flow through
H	Heavy chain
HC	Healthy controls

HisRS	Histidyl-tRNA synthetase
HLA	Human leukocyte antigen
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
IBM	Inclusion body myositis
IC	Immune complex
ICSR	Immunoglobulin isotype class-switch recombination
IC_{50}	Inhibition concentration
iDC	Immature dendritic cells
IFN	Interferon
Ig	Immunoglobulin
IIM	Idiopathic inflammatory myopathies
IL	Interleukin
ILD	Interstitial lung disease
K(d)	Affinity constant
L	Light chain
Lys	Lysine
MAA	Myositis-associated autoantibodies
MHC	Major histocompatibility complex
MCoTI-II	Momordica Cochinchinensis trypsin inhibitor II
MS	Mass spectrometry
MSA	Myositis-specific autoantibodies
MTX	Methotrexate
MZ B cells	Marginal zone B cells
NET	Neutrophil extracellular trap
NK	Natural killer cell
NM	Necrotizing myopathy
NMR	Nuclear magnetic resonance
NSAID	Non-steroidal anti-inflammatory drug
PAD	Peptidylarginine deiminase
PAMP	Pathogenic-associated molecular pattern
PG	*Porphyromonas gingivalis*
PM	Polymyositis
PTM	Post-translational modification
PTPN22	Protein tyrosine phosphatase non-receptor type 22
TLR	Toll-like receptors
Treg	Regulatory T cells
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SE	Shared epitope
SF	Synovial fluid
SFTI-1	Sunflower trypsin inhibitor-11
SHM	Somatic hypermutation
S1[Cit/Arg573]fib(566-580)	SFTI-1-Cit/Arg fibrinogen peptide (α chain amino acid residues 580-600)
S2[Cit573,Arg575]fib(566-580)	SFTI-1-Cit fib peptide mutant 2 (α chain amino acid residues 580-600)
S3[Cit573;Arg575]fib(566-580)	SFTI-1-Cit fib peptide mutant 3 (α chain amino acid residues 580-600)
TCR	T cell receptor
TG	Trigeminal ganglia
TRIM21	Tripartite motif containing 21 (Ro52)
TNF-α	Tumor necrosis factor alpha
V	Variable domain

INTRODUCTION

The immune system has evolved to protect our body from potential threats such as microbes, viruses and parasites. It encompasses an orchestra of components that should be in homeostasis in order not to react to self-molecules and simultaneously should be able to defend us from external threats. Occasionally, reactions to self-components arise and chronic inflammation develops leading to pathogenic autoimmune diseases. From an evolutionary perspective chronic autoimmune inflammatory diseases are considered to be fairly new in history because the life expectancy of our ancestors was shorter. However, in the present time, ~5% of the world population suffers from autoimmune disorders which increases the burden of such ailments (1-5). This PhD thesis intends to provide additional knowledge to the mechanisms that govern a pathogenic autoimmune state and suggests new approaches for the treatment of conditions such as rheumatoid arthritis and myositis.

1. Innate immunity

The immune system employs several different mechanisms that aim at protecting the host from invading pathogens. The first lines of defense pathogens encounter when attempting to invade the host are physical and biochemical barriers such as skin, lung, gut, eyes, nose and oral cavity (6). When pathogens invade such obstacles, tissue-resident **macrophages**, **dendritic cells** (DC), and to some extent mast cells are the first elements encountered (7-9). Upon stimulation, and with the purpose to clear away the invading agent, the resident innate immune cells phagocyte the pathogen and produce an array of **inflammatory mediators** such as: cytokines (e.g. tumor necrosis factor α, TNF-α; interleukin (IL) 1, IL-1 and IL-6) (10, 11), chemokines (e.g. chemokine C-C motif ligand 2, CCL2 and chemokine CXC motif ligand 8, CXCL8) (12, 13), eicosanoids and vasoactive amines (14). The inflammatory mediators released lead to increased vasodilation and permeability of the endothelia which in turn facilitates the passage of recruited neutrophils and monocytes into the infection site. The endothelial cells increase the expression of cell-adhesion molecules that support leucocytes adherence, hence promoting the passage through the blood vessel to the infected site (15-19). The accumulation of these inflammatory cells, serum proteins (e.g. complement system, C-reactive protein) and fluid into the infected tissue leads to increase heat, redness, swelling, and pain which translates the initiation of the inflammatory response. When the inflammatory process is not properly resolved it may lead to chronic inflammation, a characteristic feature of rheumatoid arthritis (RA) for example.

Macrophages, DC as well as neutrophils utilize soluble and membrane-bound receptors that recognize pathogenic-associated molecular patterns (**PAMPs**), not present in the host like: Toll-like receptors, nucleotide-binding oligomerization domain-like receptors, cytosolic retinoic-acid inducible gene-I, C-reactive protein, mannose binding lectin and C-type lectin receptors (6, 20-22). The usage of receptors that recognize PAMPs enables the innate immune system to recognize different pathogens and further permits the distinction between self and non-self structures (23, 24).

Cell infection by viruses is typically characterized by the production of a group of cytokines termed **interferons** (IFNα and IFNβ). **Plasmacytoid dendritic cells** (pDC, a different type of DC also involved in recognition of pathogens, specifically viruses, and

induction of primary immune response) produce higher levels of IFN in comparison to other cell types. The expression of TLR7 and TLR9 (internal sensors of viral nucleic acids) contributes to pDC efficiency at secreting IFN. IFNα and IFNβ are secreted by virus-infected cells and will generate a status of virus-resistance in uninfected host cells by impairing the capacity of the virus to replicate within the cell. By inducing the expression of major histocompability complex (MHC) I molecules in non-infected cells, IFN promotes resistance of those cells to viral infection. In addition, and also through a MHC-I related mechanism, IFN renders the newly infected cells increased susceptibility to cytotoxic lymphocytes (CD8 T cells) and thereby to be killed. IFN is also capable of activating natural killer (NK) cells that will consequently eliminate the virus-infected cells (25-28).

Natural killer cells are considered part of the innate immune system, although being derived from a common lymphoid progenitor as other lymphocytes (B and T cells). IFN and macrophages-derived cytokines induce NK cells activation, thus, NK cells are also very important for restraining viral infections at an early stage. NK cells bind viral-infected and tumor cells through specific surface receptors and once bound induce cell death by releasing cytotoxic granzymes and perforin(s). NK cells were recently described to also respond to bacteria-infected cells (29-32).

Platelets are involved in blood clotting which generates a physical barrier that avoids the spread of the infection. They rapidly move to the site of infection and accumulate in abundant number. Platelets express several innate immunity related receptors (e.g. TLR), act as granulocytes releasing anti-microbial substances and secrete inflammatory mediators important in the first stage of the inflammatory process (33).

Recent research showed the presence of **tissue-resident T cells** (e.g. innate lymphoid cells) which together with macrophages and DC continuously patrol the body for external threats. These cells participate in immune-regulation, repair and homeostasis (34). T cells were also shown to be present in healthy human skin, upholding immune surveillance (35).

2. Acquired immunity

When pathogens escape innate immunity, the host defense recruits more specialized components and the adaptive immune response (or acquired immunity) is initiated. At this point the innate immune system is crucial in order to make the transition to acquired immunity. B cells, macrophages and especially DC are 'professional' **antigen presenting cells** (APC) that bridge the two immune responses/immunities. Memory (capacity to, upon a new encounter, respond to the same antigen more rapidly and effectively) and specificity to antigens are important characteristic of the adaptive immunity. T and B cells are key players at this point since they express highly variable antigen specific receptors (T cell receptor, TCR and B cell receptor, BCR). The diversity of TCR and BCR available to recognize a wide range of antigens is achieved through gene rearrangements. Each lymphocyte recognizes one antigen that has been taken up by APCs at the site of infection and presented in secondary lymphoid organs. Upon encounter with the antigen, lymphocytes rapidly proliferate and produce a large number of cells of certain specificities (clones) capable of mounting an immune response against that particular antigen (clonal selection) (22). Certain cells of these

clones further differentiate into memory cells, capable of rapidly and efficiently respond to a secondary encounter.

2.1. T cells

Antigen presenting cells engulf and process antigens which will be presented to **$CD4^+$ T cells** through the MHC class II using the CD4 molecule as a coreceptor stabilizing the interaction. On the other hand, **$CD8^+$ T cells** are activated by MHC class I molecules presenting virally transcribed antigens or intracellular components, using the CD8 molecule as a coreceptor. T cells are generated in the bone marrow and migrate to the thymus for maturation into naïve $CD4^+$ or $CD8^+$ T cells. Thereafter, T cells circulate to secondary lymph nodes where they encounter APC expressing cognate MHC-peptide complexes. Each T cell of a particular clone expresses one specific TCR that will bind one specific pathogen-derived peptide presented by an MHC-molecule on the APC. Upon antigen presentation, and depending on the cytokine milieu, T cells will differentiate into different subsets, which in turn will secrete different effector cytokines and perform distinct biological functions. Mature DCs, which have encountered PAMPs and presented the antigens to T cells, also express surface co-stimulatory molecules important for an effective T cell proliferation and differentiation. Some examples of T cell populations are T helper (T_H) cells such as T_H1, T_H2 and T_H17, cytotoxic T cells, and regulatory T cells (T reg) (22, 24, 36, 37). B cells need T cell help to become activated in order to produce antibodies for the host defense. T_H cells also provide signals for improvement of macrophages phagocytosis capacity. After the antigen has been eliminated a group of T cells remains (memory T cells) providing long lasting immunity (38-43).

2.2. B cells

B cells are produced in the bone marrow (where they undergo selection) and are further matured in secondary lymphoid organs. Mature B cells are divided in three main subgroups: follicular B cells, B1 cells and marginal zone B cells. The three subsets have different locations, cell surface markers, antigen specificity and threshold for activation. **Marginal zone (MZ) B cells** are located in the spleen and act on blood-borne pathogens. The antibodies secreted by MZ-derived plasma cells display low affinity to the antigens. **B1 B cells** are found in the peritoneal and pleural cavities and in mucosal sites where they survey environmental-derived pathogens. Both MZ B cells and B1 B cells have the capacity to respond in a T-cell independent way. The most abundant subset of B cells is the population of **follicular B cells** that migrate to lymphoid tissues and upon antigen encountering and interaction with T follicular helper cells and follicular dendritic cells will form germinal centers (GC). During this process, antigen B cell receptors undergo somatic hypermutation (SHM) and Immunoglobulin (Ig) isotype class-switch recombination (ICSR) and ultimately differentiate into high-affinity long-lived antibody-secreting cells (**plasma cells**) or memory B cells. **Memory B cells** are important to initiate the secondary immune response. Upon meeting the same antigen memory B cells drive a faster and more efficient response, producing high affinity antibodies (22, 37, 44, 45).

The processes of V(D)J recombination (V, variable; D, diversity; J, joining gene segments), SHM and ICSR during BCR maturation are important to produce highly diverse antibodies with improved affinity to respond to antigens. This diversity is acquired through two main

stages: first, BCR diversity takes place in the bone marrow in an early stage of B cell development and independent of antigen. During this period **recombination of V(D)J segments** of the heavy (H) and light (L) chain occurs. The heavy chain is assembled by recombination of the J_H and D_H segments, followed by J_HD_H-V_H rearrangement. Subsequently, the light chain is produced by recombination of VJ segments. Upon VDJ rearrangement immature B cells express heavy chain of μ type, which leads to expression of IgM on the surface and subsequently, mature B cells express heavy chains of μ and δ type leading to IgM and IgD appearance on the surface. Recombination-activating genes (RAG) 1/2 are important mediators of V(D)J genes rearrangement. In addition, the enzyme terminal deoxynucleotidyl transferase adds extra diversity by inserting nucleotides between V_H and D_H and between D_H and J_H junctions. The secondary source of diversity that gives rise to antibodies with multiple reactivities occurs in an antigen and T cell-dependent manner once immature B cells have migrated from the bone marrow to the spleen, lymph nodes and other lymphoid tissues. The follicular B cells that present higher affinity for the antigen will generate germinal centers (GC). Within the GC **somatic hypermutation** (SHM) takes place whereby point mutations are inserted into the rearranged variable regions of the BCR. These mutations influence the avidity and specificity for the antigens. The B cells expressing high-affinity BCR after SHM survive and differentiate into memory B cells or long-lived plasma cells, which will eventually secrete high affinity antibodies. **Immunoglobulin (Ig) isotype class-switch recombination** (ICSR**)** is an important process employed to further diversify the antibody repertoire in an immune response. During class switch recombination the original constant region (C) of the BCR (Cμ, IgM) is substituted by the constant region of a different isotype (Cγ, Cα and Cε).Thus, the isotype of the substituted heavy chain C region will dictate the antibody class (IgG, IgA or IgE) produced and therefore the effector function it entails (Figure 1, Table 1) (6, 22, 45, 46). Similarly to the initial gene rearrangement of the BCR which is dependent of RAG, SHM and class switching are dependent of the expression of activation-induced cytidine deaminase (AID) in activated B cells. Throughout these maturation processes B cells undergo tolerance checkpoints whereby anti-self reactivity is eliminated (Figure 2).

2.3. Antibodies

Antibodies are tetramers composed of two light chains (L) and two heavy chains (H, Figure 1). The variable domain (V) of an antibody contains hypervariable complementary determining regions, important for antigen specificity and variety. The antigen binding sites, located in the variable domains, adapt to the three dimensional structure of an antigen when both parts come in contact. Antigen and antibody are complementary to each other and connect through non-covalent bonds, i.e., van Der Waals, hydrophobic and electrostatic interactions and hydrogen bonds. The constant regions of the heavy chain and light chain determine the isotype and sub-class of the antibody. The different antibody classes are listed in Table 1 and the basic structure of an antibody is illustrated in Figure 1 (6, 47).

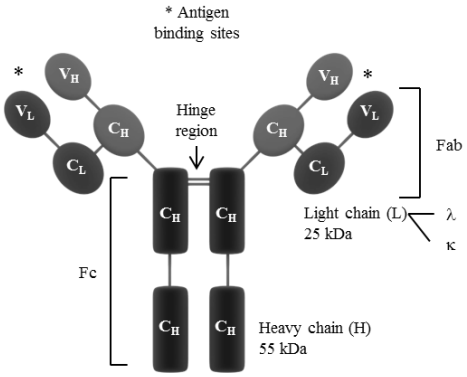
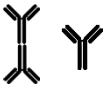


Figure 1 Basic structure of an antibody. The typical structure of an antibody allows it to perform two functions: recognition/binding of antigens by the variable domains V_L and V_H, and engagement of several immune effector functions through binding of the Fc part to Fc receptors on other cells (e.g. phagocytes, NK and T cells) and via binding and activation of the first component of the complement system (C1q). The four polypeptide chains are united through disulfide bridges and the tertiary structure is kept by covalent bonds and non-covalent interactions. *C, constant; V, variable; L, light; H, heavy; Fc, fragment crystallizable region; Fab, fragment antigen-binding.*

Table 1 Antibody classes and corresponding characteristics.

Isotypes	Structure	Characteristics
IgG		IgG is divided in four subclasses (IgG1-4) and constitutes ~75% of total serum Igs. The average concentrations for each subclass in circulation are 9, 3, 1 and 0.5 mg/mL, respectively. Molecular weights vary between 146-170 kDa. The half-life of IgG1 and 4, 2, and 3 is 21, 20 and 7 days, respectively.
IgA		IgA predominantly exists in mucosal surfaces (70-95%) but constitutes 15-20% of serum Ig. It can exist as monomers (IgA1 and 2, 160 kDa) or as a dimer (secretory IgA, 385 kDa). IgA1 is the most abundant subclass (3 mg/mL), followed by IgA2 (0.5 mg/mL) and secretory IgA (0.02 mg/mL). The half-life of IgA is 6 days.
IgM		IgM constitutes ~10% total Ig pool and is usually secreted as a pentamer of 970 kDa (1.5 mg/mL). It is the first antibody to be generated during B cell development. The half-life of IgM is 10 days.
IgE		IgE is a monomeric Ig of 188 kDa that binds with high affinity to specific Fc receptors on basophils and mast cells via its Fc-part. It is involved in allergic reactions and protection against helminth parasites. Serum concentration is below <0.05 µg/mL and the half-life is 2 days.
IgD		IgD constitutes <1% total serum Igs. A transmembrane monomeric form (184 kDa) can be found in mature naïve B cells and it acts as an antigen-specific receptor. With an average concentration of 30 µg/mL its half-life is 3 days.

Antibody-antigen interactions are reversible. The strength of the bond between them is defined as **affinity** and by applying the Law of Mass action (model 1:1, antigen:antibody) it is possible to determine how strong the bond is. The Law of Mass action states:

$$[\text{Antibody}] + [\text{Antigen}] \rightleftharpoons [\text{Antibody x Antigen}] \quad K = \frac{[\text{Antibody}][\text{Antigen}]}{[\text{Antibody x Antigen}]}$$

The affinity of an antibody is translated by the dissociation constant K given in Molar units, at equilibrium. The closer K is to zero, the higher the affinity. Antibodies with $K < 10^{-7}$ M are said to bind antigens with high affinity, whereas antibodies with $K > 10^{-7}$ M are considered low affinity. Although the binding affinity is commonly used to describe these types of interactions, antibodies contain two or more antigen binding sites and antigens are usually multivalent, therefore the term **avidity** (which considers antibody and antigen valence) is a

more accurate and relevant approach to define the binding strength. This concept requires more advanced mathematical models (6, 45, 48).

Plasma-cell secreted antibodies remove and destroy pathogens by modulating immune functions through a variety of mechanisms: 1) Generation of immune complexes (IC) that will activate the complement system in order to lyse and opsonize bacteria. Immune complexes are composed by a group of antibodies that binds to a pathogen and engage components of the complement pathways; 2) Antibodies opsonize pathogens present in the extracellular space by binding to Fc receptors on phagocytes; 3) Antibodies recognize and neutralize a pathogen or bacterial toxins thus preventing them from attaching to cells (inactivation), and 4) antibodies bind pathogen-derived antigens on cell surfaces, such as virally encoded proteins, and induce antibody dependent cellular cytotoxicity (ADCC) by interacting with Fc receptors on NK cells, CD8 T cells and myeloid cells (6, 22).

3. Mechanisms of central and peripheral tolerance

Immunological tolerance, i.e., state of unresponsiveness for certain antigens that have been previously encountered by the immune system, comprises several mechanisms that are important to avoid inadequate immune reactions. For instance, tolerance is essential to prevent reactivity against self-components as well as against innocuous air-borne and food antigens. A deficient regulation of tolerance may lead to pathogenic autoimmune processes. Figure 2 illustrates the various steps of T and B cell tolerance (44, 45, 49).

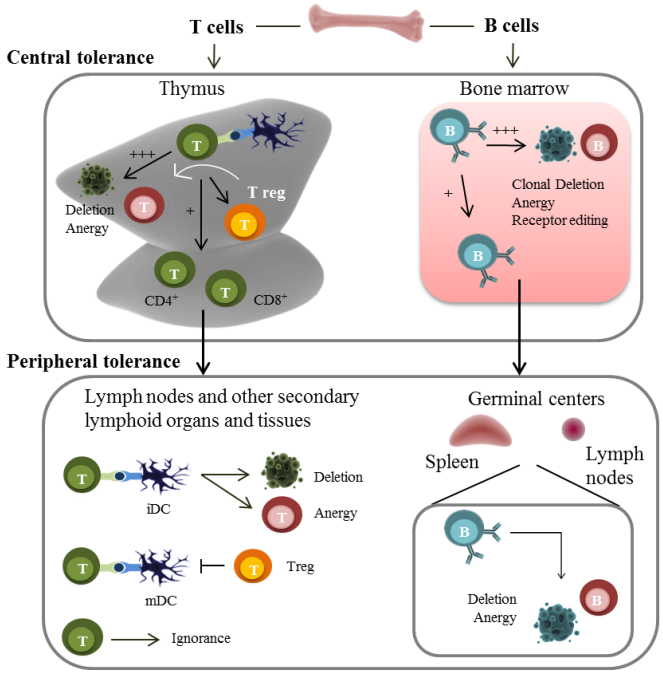


Figure 2 Mechanisms of central and peripheral tolerance of T and B cells. Immature T cells (T) enter the thymus and the T cell receptor (TCR) undergoes rearrangement. T cells with a functional TCR will express CD4 and CD8 and those CD4/8 cells that bind the MHC with low affinity (+) are selected. The T cells that bind too strongly to the MHC (+++) will be eliminated by apoptosis (deletion). Autoreactive T cells also die by deletion. Regulatory T cells (Treg) are generated in the thymus in order to additionally control auto-reactivity. The thymus expresses a large amount of antigens which contributes to the negative selection of T cells (50, 51). In the periphery, T cells that escaped central tolerance undergo several steps of selection upon presentation of antigens by the MHC. Naïve T cells undergo anergy (do not become activated) or are deleted in peripheral organs if DCs process and present self-antigens through MHC molecules in the absence of co-stimulatory molecules (e.g. CD80 and CD86), i.e. in the absence of infection (PAMPs) or inflammation (damage associated molecular patterns: DAMPs). Peripheral tolerance to self-reactive T cells is also mediated by Tregs. Auto-reactive B cells are generated because V(D)J rearrangements and additional receptor editing are random. B cells of low affinity (+) are positively selected whereas those displaying high affinity (+++) to self-antigens are eliminated by deletion or undergo receptor editing in the bone marrow in order to express new non-autoreactive BCRs. Autoreactive immature B cells may enter a state of anergy if lacking the signals for proper activation. If autoreactive B cells reach peripheral tissues they also may enter in a state of anergy or are eliminated in post-germinal centers (22, 44, 45). *iDC immature or resting DC; mDC, mature DC; +++, high affinity; +, low affinity.*

4. Autoimmunity and pathologic autoimmunity

Simply put, autoimmunity arises due to tolerance failure. Albeit autoimmunity is a normal occurrence in vertebrate life, the progression to autoimmune disease requires several events to take place in parallel and an appropriate microenvironment. Therefore, an autoimmune disease is defined as a condition where the constant activation of the adaptive component of the immune system, translated by the recurrent presence of autoreactive T cells and/or autoantibodies when the infection is no longer present, leads to tissue injury (52).

4.1. Criteria to define autoimmune disease

Noel Rose and Constantin Bona established several criteria for the definition of autoimmune diseases based on Witebsky's previous claims (53). Table 2 below illustrates the different evidence-based autoimmunity definition.

Table 2 Description of the different evidences employed to define the concept of autoimmune disease

Type of evidence	Definition
Direct	The characteristic lesions of an autoimmune disease are generally replicated through the direct transfer of autoantibodies. Between humans: autoantibodies cross the placenta and induce disease in the fetus. For example, the transfer of anti-thyroid stimulating hormone receptor antibodies causes hyperthyroidism; transfer of anti-Ro antibodies causes congenital heart block in the infant. Experimentally, autoimmune disease can be reproduced in animals after the transfer of human serum or affinity purified human antibodies. For instance, transfer of human Ig from patients with pemphigus into new-born mice leads to characteristic skin lesions; administration of anti-acetylcholine receptor antibodies to mice causes muscle weakness, characteristic of myasthenia gravis.
Indirect	Recognition of causal autoantigen and reproduction of the disease by immunization of animals with the identified autoantigen: thyroiditis is induced by the antigen thyroglobulin; immunization with acetylcholine receptor causes myasthenia gravis. Cell-transfer autoimmune disease: induction of experimental allergic encephalomyelitis by $CD4^+$ T cells specific for myelin basic protein (MBP); induction of arthritis using collagen type II-specific T cells. Genetically-induced models: New Zealand black (NZB) mice develop autoimmune hemolytic anemia. Autoantibodies purified from NZB mice induce hemolytic anemia in healthy mice. Tight-skin (TSK) mice develop scleroderma-like syndrome. Monoclonal antibodies from TSK mice and scleroderma patients present similar reactivity against the antigen topoisomerase I. Additional indirect evidence is given when it is possible to isolate autoantibodies or auto-reactive T cells from the target organ: isolation of anti-DNA autoantibodies from kidneys of patients with SLE; from the thyroid of patients with Grave's disease it's possible to isolate cytotoxic T cells.
Circumstantial	If direct or indirect evidences are lacking an autoimmune disease can be defined considering several common indicators. For instance, if the patient has family history, simultaneous presence of other autoimmune diseases, high titers of autoantibodies, infiltrates in target organ and improvement of certain symptoms after being medicated with immunosuppressants.

Approximately 80 different autoimmune diseases are described in the literature. These entities span over a broad range of affected organs. On one side of the spectrum the autoimmune response targets a specific organ (E.g. Hashimoto's thyroiditis, pernicious anemia and Addison's disease); SLE lies in the opposite side of the spectrum and it is known

as a non-organ specific or systemic disease. Rheumatoid arthritis and the idiopathic inflammatory myopathies (IIM, such as dermatomyositis, DM and polymyositis, PM) are located in the mid part of the spectra because several organs can be affected although not in such a wide range as for SLE (6).

4.2. General features of pathologic autoimmunity

Although autoimmune disease can present different phenotypes, some features are common and collectively characterize a pathologic autoimmune event.
Virtually all autoimmune diseases have a **genetic component**. The most striking association lies on the human leukocyte antigen (HLA) gene family. HLA-DRB1 shared epitope (SE) alleles are closely associated with RA (54), and specifically with the presence of autoantibodies in sera and synovial fluid (SF) (55, 56). Similarly, HLA-DR phenotype has been associated with a subset of IIM patients having anti-histidyl transfer RNA synthetase (HisRS) antibodies (aJo1) (57, 58). HLA has also been associated with other autoimmune diseases. HLA-DRB1*03:01 was found to correlate with SLE (59), type I diabetes (60) and scleroderma (61).

Environmental factors are also considered a major contributing risk factor for autoimmune diseases. Smoking, chemicals, UV light, drugs and infections are recognized environmental triggers that when exposed to susceptible organs (e.g. mucosal sites such as the lung) may initiate an immune response. Smoking, for example, has been associated with anti-citrullinated protein/peptide antibodies (ACPA)-positive RA as well as with aJo1-positive IIM. Moreover, evidence shows a correlation between HLA-DRB1 alleles and smoking in both these seropositive disease populations (57, 62). Viral and microbial infections have also been debated as an etiological agent, although no solid proofs have been provided yet. Some concrete examples of infections seemingly associated with RA are those induced by *Porphyromonas gingivalis* (PG) and Epstein-Barr virus. Patients with periodontitis and infected with PG have a higher likelihood of developing RA, whereas seropositive (ACPA and rheumatoid factor, RF) RA patients are more likely to have moderate to severe periodontitis (4, 63, 64). Coxsackie viruses were suggested to be involved in the initiation of IIM (more specifically PM and DM) (65-67). A flagrant example of an autoimmune condition induced by exposure to a drug is the statin-induced necrotizing myopathy. These patients develop severe muscle weakness and autoantibodies against 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), the target of statins, after medication with statins. However, it is still under debate whether anti-HMGCR antibodies are a true autoimmune response or just an epiphenomenon (68, 69).
The fact that there is a sex bias in a large percentage of autoimmune diseases raises the question whether **hormones** play a role in the aetiology of such conditions (44).

4.3 Autoantigens and autoantibodies

When the immunological tolerance to self-antigens fails an autoimmune process initiates. One of first manifestations is the occurrence of autoantibodies that arise as an immune response towards those self-antigens (70, 71). Autoantibodies target different types of molecules namely, proteins, nucleic acids and lipids that can be located in the nucleus or cytoplasm, membrane surface or in the extracellular environment. In some autoimmune

diseases the affected tissues or organs express unique antigens that become the specific target of the immune response (e.g. MBP in multiple sclerosis). In other cases, autoantibodies are raised against ubiquitously expressed proteins (topoisomerase 1 in scleroderma, HisRS in IIM or histone 4 in RA are some examples). Some currently known autoantibodies are listed in Table 3. The reasons why certain tissues (lung, muscle, joint) are targets of an autoimmune attack, why certain molecules become autoantigenic and whether autoantibodies intervene in the initiation and development of an autoimmune disease are active areas of research (72, 73).

Table 3 Autoantibodies used as diagnostic and prognostic biomarkers (Table adapted from 'The autoimmune disease fifth edition', chapter 77 by Marvin J. Fritzler) (44)

Autoantibody	Antigen	Disease
ACPA	Citrullinated proteins/peptides	RA
RF	Fc region of IgG	RA
Anti-aaRS	Aminoacyl-transfer RNA synthetases	Myositis; ASS
Anti-DNA	DNA packed around histones	SLE
Anti-Ro52 (SSA)	Tripartite Motif Containing 21	SLE; Other SAD
Anti-Ro60 (SSA)	Ribonucleoprotein from TROVE (Telomerase, Ro and Vault) domain family member 2	SLE, SjS
Anti-La (SSB)	3' poly(U) terminus of nascent RNA polymerase III transcripts 48 kDa binding protein	SLE, SjS
Anti-Scl-70	Type I DNA Topoisomerase	SSc
Anti-CENPB	Centromere protein B	SSc
Anti-Sm	Complex of small nuclear ribonucleoproteins	SLE
Anti-Pm-Scl	Exosome complex	SSc
ANCA	Myeloperoxidase (p-ANCA) and proteinase 3 (c-ANCA)	Vasculitis
ACA	Cardiolipin	APS; SLE
Anti-Lupus anticoagulant	Phospholipids	APS; SLE
Anti-tTG	Tissue transglutaminase	CD
Anti-TPO	Thyroid peroxidase	HT
Anti-AChR	Acetylcholine receptor	MG
Anti-desmoglein	Desmoglein (cadherin-like transmembrane glycoprotein)	PV
AMA	Mitochondria	PBC

RA, rheumatoid arthritis; ASS, anti-synthetase syndrome; SLE, systemic lupus erythematosus; SAD, systemic autoimmune diseases; SjS, Sjögren's Syndrome; SSc, Systemic sclerosis or scleroderma; APS, anti-phospholipid syndrome; PA, pernicious anemia; CD, celiac disease; HT; Hashimoto's thyroiditis; MG, myasthenia gravis; PV, pemphigus vulgaris; PBC, primary biliary cirrhosis.

4.3.1 Autoantibody generation

Defects in the tolerance checkpoints at a central level are known to occur in autoimmune diseases (74, 75). However, it appears that somatic hypermutation in the GCs in secondary lymphoid tissue may also be important for the generation of autoreactive B cells (Figure 1). Memory B cell-derived autoantibodies in systemic autoimmune diseases such as SLE and RA are usually hypermutated and class-switched, which shows their participation in GC processes (74, 76-81). Autoantibody diversification and production can also occur in ectopic structures in non-lymphoid target organs such as the lung and synovial tissue in RA and kidney in SLE (72, 82-84).

One mechanism by which loss of tolerance takes place is when self-proteins undergo modifications which lead to generation of new epitopes that are not expressed in the thymus,

and thus have not been engaged in negative selection and tolerization. After the protein synthesis, the amino acids undergo covalent alterations designated **post-translational modifications (PTM).** In a normal setting, PTM are important regulators of the function of a protein namely by affecting the activity, interactions, subcellular localization, stability and proteolytic degradation. Among hundreds described, phosphorylation, citrullination, ubiquitination, glycosylation, proteolysis and acetylation are some common examples of PTMs (85). These processes affect the primary, secondary and tertiary structure of proteins and in certain contexts such as inflammation and cell death (apoptosis, necrosis and lymphocyte granule pathway) may affect antigen selection and processing and thereby installing an autoimmune process (86-88).

During **apoptosis** (for example in SLE) autoantigens were found to cluster in apoptotic blebs on the cell surface, promoting the release of intracellular antigenic targets such as DNA and ribonucleoproteins (89). Normally, these apoptotic blebs are taken up by phagocytes with no major consequences. However, in a context of defective apoptotic clearance as observed in autoimmune conditions, intracellular antigens accumulate in the extracellular milieu and may drive autoantibody generation. The process of programed cell death stimulates the formation of different PTMs as is the case of **citrullination** which is the PTM known to have the strongest association with RA. This PTM will be described in more detail in the section 5.4.1 of the introduction.

In the case of myositis the **lymphocyte granule pathway** seems to be the major form of cell death. Several myositis related autoantigens were shown to be susceptible to cleavage by proteases contained in cytotoxic lymphocyte granules. Upon cleavage by, for example, the serine protease **Granzyme B (GrB)**, novel fragments are formed which renders the antigen immunogenic properties that may lead to autoantibody generation (90-92).

An alternative type of cell death occurs when neutrophils release chromatin webs (neutrophil extracellular traps, NETs) which trap bacteria – **NETosis**. Besides microbial peptides, NETs were demonstrated to expose pro-inflammatory mediators (e.g. IL-17), and post-translational modified autoantigens such as citrullinated, methylated and acetylated histones (in RA and SLE, respectively). Moreover, NETosis was shown to be enhanced in RA and to associate with the presence of ACPA and pro-inflammatory molecules. These findings suggested that defective NETosis in RA may contribute to the autoimmune process and disease pathogenesis (87, 93-97).

The importance of autoantigens as disease drivers can be discussed from another viewpoint. The main target organ of an autoimmune response may not always be the main source of the autoantigen, initially. A compelling example is the enzyme HisRS and the muscle in IIM. HisRS is one of the most common autoantigens in IIM (described in detail in section 6.2.1 of the introduction) whereby the muscle is the major target organ. Casciola-Rosen and co-workers (98) clearly showed that HisRS is highly expressed in regenerating muscle fibers in IIM whereas only very low levels are detected in healthy differentiated muscle fibers. The fact that HisRS is overexpressed in this particular condition suggests that it is the regenerating muscle fiber rather than the healthy muscle fiber that is the target of an autoimmune response. In addition, HisRS was suggested to act as a tissue-chemoattractant since it was shown to activate immature DC and naïve lymphocytes via C-C chemokine receptor type 5 (CCR5)

(99). These observations provide support for the crucial role of autoantigens as disease drivers in a pathogenic autoimmune situation (90, 92, 100).

4.3.2 Autoantibodies in diagnosis and pathogenic mechanisms

Sensitivity, specificity, positive or negative predictive values and receiver operating characteristic (ROC) curves are important concepts in the analysis of diagnostic tests. The term *sensitivity* translates the proportion of patients that test positive due to the fact that they have the disease thus, the patient is correctly identified by the test. The probability an individual has of presenting the disease because that subject tested positive, is denoted as the *positive predictive value*. The term *specificity* is the proportion of individuals that test negative for the disease because they do not have the disease. The probability an individual has of not having the disease because the test was negative, translates the *negative predictive value*. The *ROC* curves give a correlation between the sensitivity and specificity (101, 102).

Disease definition and classification. Autoantibodies are good disease markers and are commonly used for diagnostic purposes. Laboratory techniques such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence or immunoprecipitation are regularly used to detect autoantibodies in patient serum. Anti-mitochondrial antibodies (AMA) are included in the diagnostic criteria of primary biliary cirrhosis. Around 90-95% of patients have AMA (103). The presence of anti-parietal cells or anti-intrinsic factor (important for Vitamin B12 absorption) autoantibodies together with atrophic gastritis and vitamin B12 levels translates the diagnosis of autoimmune gastritis (also termed pernicious anemia) (104, 105).

Disease activity and prediction. The presence of autoantibodies may give insights on disease activity, severity and prognosis and the severity of a chronic inflammation is often reflected by the intensity of the immune response. As autoantibodies are a consequence of the pro-immune setting, they can in some cases be used as indicators of disease activity e.g. anti-DNA and anti-Jo1 antibodies (predicting rate of progression). In many autoimmune diseases autoantibodies are present several years before clinical onset. In type 1 diabetes and thyroiditis, which are organ-specific conditions, circulating autoantibodies have been detected before the destruction of hormone-secreting cells. The autoantibody titers in these cases may reflect disease severity (106, 107). The occurrence of autoantibodies is sometimes associated with the development of a more aggressive disease, as observed for ACPA+ RA patients having more severe erosions and bone loss compared to ACPA- RA. In addition, ACPA may also be detected long before the first clinical symptoms, up to 10 years, and affinity maturation, isotype switching and increased epitope spreading take place close to diagnosis (further details in section 5.4.1.1 of the introduction). The ability to predict a disease, especially in those individuals with a long pre-clinical stage, is essential for prevention. These subjects may avoid certain environmental risk factors, known to have a direct correlation with that specific autoimmune disease. Individuals with pre-existing autoimmunity may be treated during the pre-clinical stage (105, 108).

In addition, autoantibodies may be useful molecular tools since they can be employed to isolate the cognate molecules and therefore study the molecular mechanisms behind the disease. The role of small nuclear ribonucleoproteins (snRNP) in splicing-messenger RNA has been possible to study due to the use of anti-snRNP antibodies to immunoprecipitate

snRNP (109). Likewise, anti-centromere autoantibodies, characteristic of scleroderma patients, were employed to elucidate the structure and function of kinetochore (protein involved in chromosome condensation and cell division) (110).

Although some autoantibodies are harmless (e.g. natural autoantibodies), others have proven to be **pathogenic**. The affinity/avidity, epitope specificity, class/sub-class and glycosylation pattern of an autoantibody influence its pathogenic effects. Several mechanisms defined above for antibodies also apply in this particular situation (111): **(1) Opsonization and phagocytosis**- Autoantibodies bound to target cells activate mononuclear phagocyte cells through Fc receptor interaction. Target cells are engulfed and destroyed (example, anti-erythrocyte antibodies in SLE and autoimmune hemolytic anemia) (112); **(2) Complement system** – Autoantibodies induce the membrane attack complex mainly via activation of the classical pathway. The complex creates a transmembrane gate and the passage of water and electrolytes disturbs the osmotic balance and consequently the cells lyse (autoimmune hemolytic anemia) (113). A deficiency in the complement system (e.g. C1q) is associated with susceptibility to develop SLE. This may be due to an impaired capacity of IC and apoptotic material clearance. Complement activation was also shown to be associated with inflammation and organ damage in SLE (e.g. C3a and C5a act as chemoattractant agents for leucocytes; renal deposition of C5b-9 releases inflammatory mediators) (114-117); **(3) Cell engagement** or **ADCC** - Autoantibodies bound to a target cell are recognized by Fc receptors on NK and $CD8^+$T cells that carry proteases-containing granules. In turn, these cytotoxic enzymes induce apoptosis of antibody-coated cells (anti-TPO autoantibodies and autoimmune thyroiditis) (118); **(4) Immune complex** - IC deposition is a characteristic feature of several autoimmune diseases. Through binding to Fc receptors or complement system, autoantibody-containing ICs promote local inflammation and consequently tissue damage (e.g. joints in RA and kidneys in SLE). Mechanistically, cell destruction takes place as described above. IC can also engage Fc receptors in NK and $CD8^+$ T cells (6, 111).

5. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disorder with an overall prevalence of 0.5 to 1% in western countries and an incidence of 2-50/100 000/per year. RA has a regional distribution, being more predominant in areas such as North America and North Europe and similarly to other autoimmune diseases women are more often affected than men. In RA the joints are targets of major damage and persistent inflammation in the synovium causes pain, joint swelling, and cartilage and bone destruction, ultimately leading to severe disability. Extra-articular involvement is often present, i.e. lung, heart, muscle, blood vessels, skin and eyes, representative of a systemic condition. An autoimmune component adds to the spectrum of RA as several autoantibodies are present in a large majority of the patients (44, 119-121). However, the immune specificities have not been clarified in detail. Furthermore, whether the antibodies are pathogenic or an epiphenomenon will be further discussed below.

5.1. Classification criteria

With the aim of distinguishing RA from other arthritides (characterized by inflammation in the joints or in other components of the musculoskeletal system) and considering the heterogenic nature of RA, two classification criteria are currently utilized: 1987 American

College of Rheumatology (ACR) (122), and 2010 ACR-European League Against Rheumatism (EULAR) (123, 124) criteria. The description of each set of criteria is listed in Table 4.

Table 4a Classification criteria for RA – The 1987 ACR criteria

Criterion	Manifestations	Score
1. Morning stiffness	In and around the joints for ≥ one hour	1
2. Arthritis in three or more joints	Simultaneous soft tissue swelling or fluid in three out of 14 joint areas: right or left proximal interphalangeal (PIP), metacarpophalangeal (MCP), wrist, elbow, knee, ankle, and metatarsophalangeal joints	1
3. Arthritis of hand joints	One or more swollen areas in a wrist, PIP or MCP joint.	1
4. Symmetric arthritis	Simultaneous bilateral involvement of the same joint areas as defined in 2	1
5. Rheumatoid nodules	Subcutaneous nodules above bony prominences, or extensor surfaces, or in juxta-articular regions	1
6. Serology	Presence of rheumatoid factor in serum	1
7. Radiographic changes	Typical for RA on posteroanterior hand and wrist radiographs. These include erosions or clear bone decalcification in or adjacent to the involved joints	1
Diagnosis: Four of the seven criteria should be fulfilled and criterion one to four should have been present for at least six weeks.		

Table 4b Classification criteria for RA – The 2010 ACR/EULAR criteria

Criterion	Manifestations	Score
A. Joint involvement	1. One large joint (shoulders, elbows, hips, knees, and ankles)	0
	2. Two to ten large joints	1
	3. One to three small* joints with or without large joint involvement (*MTP and PIP joints, second through fifth MTP joints, thumb interphalangeal joints, and wrists)	2
	4. Four to ten small joints (with or without large joint involvement)	3
	5. More than ten joints (at least one small joint)	5
B. Serology	1. Negative RF and negative ACPA	0
	2. Low-positive RF or low-positive ACPA	2
	3. High-positive RF or high-positive ACPA	3
C. Acute-phase reactants	1. Normal C-reactive protein (CRP) and normal erythrocyte sedimentation rate (ESR)	0
	2. Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	1. < six weeks	0
	2. ≥ six weeks	1
Diagnosis: Six or more of the maximum ten points. This criteria aims at early diagnosis.		

5.2. Etiology

The reason why and how RA initiates and develops is still enigmatic. However, compelling evidence suggests the interaction between genetic and environmental factors as a crucial trigger. Data from twin studies provide a good opportunity to investigate the impact that both genes and environment display on a disease. RA prevalence in twins was shown to be increased by ~15% in comparison to the general population (119, 125). More recently, MacGreggor and co-workers (126) analysed two European twin populations and reported a

higher heritability contribution of RA in the range of 60%. Several **genes** have been associated with RA. The most striking associations lie on HLA alleles, though non-HLA genes have also been reported, such as peptidyl arginine deaminase 4 (PAD4), protein tyrosine phosphatase non-receptor type 22 (PTPN22), Tumor Necrosis Factor α-Induced Protein 3, signal transducer and activator of transcription 4, chemokine (C-C motif) receptor 6 and interferon regulatory factor 5 (54, 108, 127). Interestingly, HLA-DR variants associated to RA have a common sequence of amino acids in their antigen-binding grove, termed shared epitope (SE) which may indicate that a selection of antigens is presented to T cells and consequently provides evidence for the role of adaptive immunity in the development of RA (128). Smoking is a well-established **environmental risk** factor for RA. It correlates with ACPA positivity, disease activity and extra-articular manifestations (129-132). A striking association between SE genotype and smoking has been demonstrated for the ACPA+ population of RA, and it will be further discussed in the following sections.

5.3. Pathological features

The major target organ in patients with RA is the joint. The clinical presentation of RA varies among patients, with some individuals experiencing a more aggressive disease (serious cartilage and bone loss), while others do not present erosions. Small and medium size joints are usually symmetrically affected. The systemic manifestations affect the patients in a heterogeneous way. The most common extra-articular sign is the presence of rheumatoid nodules in the juxta-articular region and in the lungs. Rheumatoid arthritis is associated with an increased risk for both cancer (lymphomas) and co-morbidities (cardiovascular disease and interstitial lung disease, ILD). Arthritic synovial joints display typical pathologic features, represented in Figure 3 (120, 121, 133, 134).

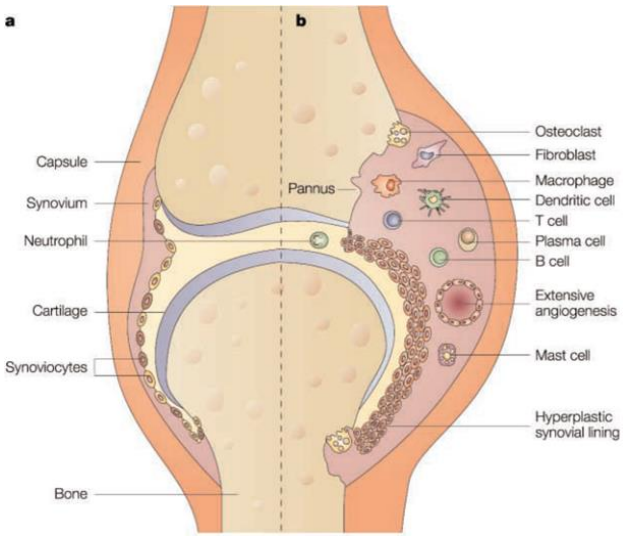


Figure 3 Illustration of a normal (a) and arthritic (b) synovial joint. (a) The synovial joints (intended to facilitate movement between adjacent bones) are surrounded by a capsule. The adjacent bones are covered by a protective thin layer of cartilage and the synovial membrane. The synovium, or synovial membrane, is composed of synoviocytes (macrophage and fibroblast derived), blood and lymph vessels and a few mononuclear cells. The synovial cavity is filled with synovial fluid (SF) containing lubricants and nutrients. The initial phase of clinical RA (b) is characterized by synovitis (inflammation of the synovium), translated by an increased number of adhesion molecules and consequent influx and activation of T and B cells, NK cells, granulocytes, mast cells, DC, plasma cells and macrophages into the synovial membrane as well as the SF. New blood vessels form and the cells from the lining layer proliferate leading to thickening of the synovium. The cartilage is degraded and the osteoclasts promote bone erosion (’pannus’). The SF contains high amounts of inflammatory cells that produce prostaglandins and oxygen-reactive species, thus contributing to the progress of inflammation and volume expansion. Fibrin deposits are found in the joint and further contribute to the ongoing inflammation. *Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery Smolen JS & Steiner G, Vol. 2(6), p.47 copyright 2003* *http://www.nature.com/nrd/journal/v2/n6/full/nrd1109.html*

5.4. Seropositive RA

The presence of circulating autoantibodies is a common manifestation of autoimmune diseases. A large subset of RA patients produces autoantibodies that target different cellular components. The antibodies are helpful in the diagnosis of RA to distinguish patients with RA from other chronic arthritis. However, if the antibodies have a role in the pathogenesis is still an enigma. To learn more about the role of autoantibodies in the pathogenesis of RA more detailed information is needed on the different reactivities that compose the autoantibodies pool (Paper I) and other characteristic features such as affinity maturation and glycosylation patterns (discussed below), which may dictate their effector functions (Paper II).

The **rheumatoid factor** (RF) was for a long time considered the only serological marker included in the classification of RA. The RF (mainly IgM but to a less extent also IgG and IgA) recognizes the Fc region of IgG and is present in up to ~80% of RA patients. Several studies have shown an association between RF and severe, destructive RA. However, RF is not specific for RA as it has been reported in healthy individuals, in patients infected with viruses and in other chronic inflammatory disorders (135-137).

Antibodies directed towards carbamylated proteins (**anti-carP antibodies**) were recently found in RA patients. These autoantibodies were detected in both the CCP+ and the CCP- RA populations (30% and 16%, respectively) and found predictive of a severe disease course (138). Recent studies show that the presence of this subset of antibodies predicts development of RA in patients with arthralgia. Similarly to RF and anti-cyclic citrullinated peptides (CCP) antibodies, anti-CarP antibodies are also detected before onset of RA. Figure 4 illustrates citrullination and homocitrullination, two of the major PTMs in RA.

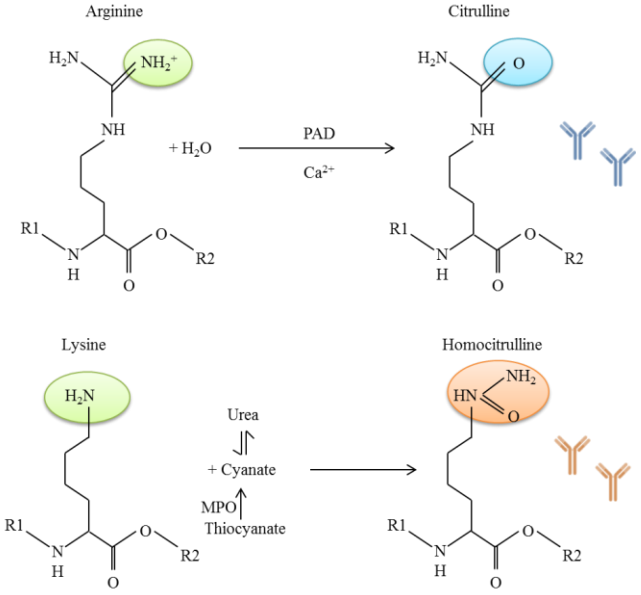


Figure 4 Representation of citrullination and homocitrullination. The conversion of arginine to citrulline by means of a peptidylarginine deiminase (Ca^{2+}-dependent enzyme, PAD) is targeted by ACPA (or anti-CCP antibodies), upper panel. Homocitrulline is produced from lysine through a chemical reaction (termed carbamylation) that requires cyanate. The cognate autoantibodies are termed anti-carP antibodies, lower panel.

Interestingly, antibodies directed against PAD4 (**anti-PAD4 antibodies**) were found to 1) occur in 36-42% of RA patients, 2) precede RA clinical onset for several years (18.1% of

individuals) and 3) to be associated both with erosive arthritis and with ILD. A positive association between the presence of these antibodies and anti-CCP antibodies was also found. Notably, anti-PAD4 antibodies recognize an epitope located in the N-terminus of the PAD enzymes that contains three RA-associated polymorphisms. In addition, anti-PAD4 antibodies were shown to cross-react with anti-PAD3 antibodies and to alter the enzyme requirement for calcium. This finding demonstrates the autoantibody's ability to institute a feed-forward loop that may be a critical step in the progression of bone erosion (139-144).

5.4.1. The case for citrullinated proteins and ACPA

Anti-citrullinated protein/peptide antibodies (ACPA) are a subset of autoantibodies that are present in up to 70% of RA patients. ACPA occur as a response to proteins in which arginine residues are converted to citrulline residues, a PTM termed citrullination or deamination (Figure 4) (145). The reduction in the net charge of the amino acid residues, as they become citrullinated, renders the proteins immunogenic features, leading to tolerance breach to previous known self-proteins.

The enzymatic reaction of protein citrullination is an important physiological process mediated by a class of calcium-dependent enzymes termed **PADs**, of which five members are described. The tissue distribution and substrate specificities differ among the five PAD members (PAD1-4 and PAD6) (145, 146). For instance, PAD1 has a fundamental role in keratinocyte differentiation, and therefore has a high level of expression in the epidermis; PAD3 is involved in hair follicle development; PAD2 is expressed in the brain (important during development of central nervous system) and in the skeletal muscle. Hematopoietic cells express both PAD2 and PAD4. PADs have been also demonstrated to be involved in the regulation of the immune system, e.g. in the formation of NETs and modulation of chemokine activity (147, 148).

An abnormal degree of protein citrullination characterizes RA. In the rheumatic joint several proteins were shown to be citrullinated (fibrinogen, vimentin, α-enolase, type II collagen (CII), histone 4, immunoglobulin binding protein and annexin). In addition, circulating antibodies targeting those citrullinated proteins have being reported (93, 149-159). Anti-citrullinated **type II collagen**, **fibrinogen**, **α-enolase** and **vimentin** antibodies composed the four best described ACPA fine-specificities and they will be further discussed in the section results and discussion (Paper I). PAD2 and 4 are expressed in the synovial joint (tissue and synovial fluid, both intra and extracellularly) (146, 151, 153, 160, 161). The differential distribution of PAD enzymes and substrate specificity may produce various citrullinated peptides that could be recognized by autoreactive T cells (160, 162). The generation of intracellular citrullinated autoantigens in RA has been suggested to take place extracellularly after cell death, because PADs need high concentration of calcium to catalyze the reaction. The intracellular concentration of calcium is too low for PAD requirements (153). More recently, protein citrullination in RA was suggested to be "cell-associated" and to occur in a high number of proteins, to which the authors coined the term "cellular hypercitrullination". Perforin and membrane attack complex (MAC) pathways were demonstrated to activate PADs and to induce hypercitrullination in synovial fluid cells (e.g. neutrophils). Apoptosis, NETosis, and autophagy/necroptosis as well as activation signals such as cytokines were not

able to induce such a great rate of citrullination further suggesting the importance of immune-mediated pore forming pathways in this process (163).

Hypercitrullination of proteins has been shown not only in RA but also in multiple sclerosis, Alzheimer's disease and to some extent myositis, which demonstrates that abnormal protein citrullination is a common phenomenon in chronic inflammatory conditions (156, 164, 165). Notably, the occurrence of autoantibodies that target citrullinated proteins, ACPA, has been shown to be specific, although not entirely exclusive, for RA as they have also been reported in other rheumatic conditions, for instance: 5 to 14% of patients with Primary Sjögren's syndrome (166-168), up to 27% and 24% of patients with myositis (167, 169) and scleroderma (170, 171), respectively, and 14% of patients with ankylosing spondylitis (167). A French case-control retrospective study identified 17 ASS patients that contain ACPA. All ACPA+ ASS patients presented arthritis in comparison to 41% of ACPA- ASS (172).

In the clinical setting, ACPA are routinely measured using a commercial ELISA based on a library of cyclic citrullinated peptides ("second generation" anti-CCP ELISA) (173, 174). Although a good diagnostic tool, anti-CCP antibodies are considered a surrogate marker for RA since CCP do not translate a 'real' human protein sequence. Studies in the past years (including paper I of this thesis) have been elucidating the nature of these autoantibodies, their reactivities and whether their autoantigens represent rheumatoid tissue targets present *in vivo* or not (56, 175-179). Knowledge on the "real" ACPA targets may lead to the design of more tailored therapies (e.g. oral tolerance) and it may provide clues for the etiopathology of RA.

A support for a role of autoantibodies in pathogenesis is that they are present before disease symptoms. Indeed, circulating ACPAs have been detected several years before RA onset, with titers and number of reactivities rising closer to the time of diagnosis (70, 176, 178, 180-184). The presence of these antibodies classifies a subset of RA characterized by worse prognosis and poor outcome (185-190). In addition, the presence of ACPA predicts the development of RA in 40 to 70% of individuals with arthralgia and helps to identify patients at risk. The sustained presence of different ACPA isotypes translates an ongoing activation of ACPA producing-B cells and the enrichment of ACPA in the joints is suggestive of a locally driven immune response (191-193). Additional evidences suggest ACPA to mediate a major role in the pathogenesis and development of RA (Figure 5). Although the joint is the major target in RA, the starting point of immune reaction is still not clear. A number of recent studies provide compelling evidence for the role of lungs as the initiating organ. But the question then remains as to why the inflammation attacks the joints. Figure 5 illustrates one current hypothesis for how ACPA+ RA is initiated, how it evolves and the major underlying molecular mechanisms (62, 70, 83, 108, 157, 177, 180, 181, 183, 184, 192, 194-208).

5.4.1.1. ACPA features

In vivo antibody responses undergo isotype switching and affinity maturation. These are important processes because they regulate the effector function of an antibody and consequently influence the immune response.

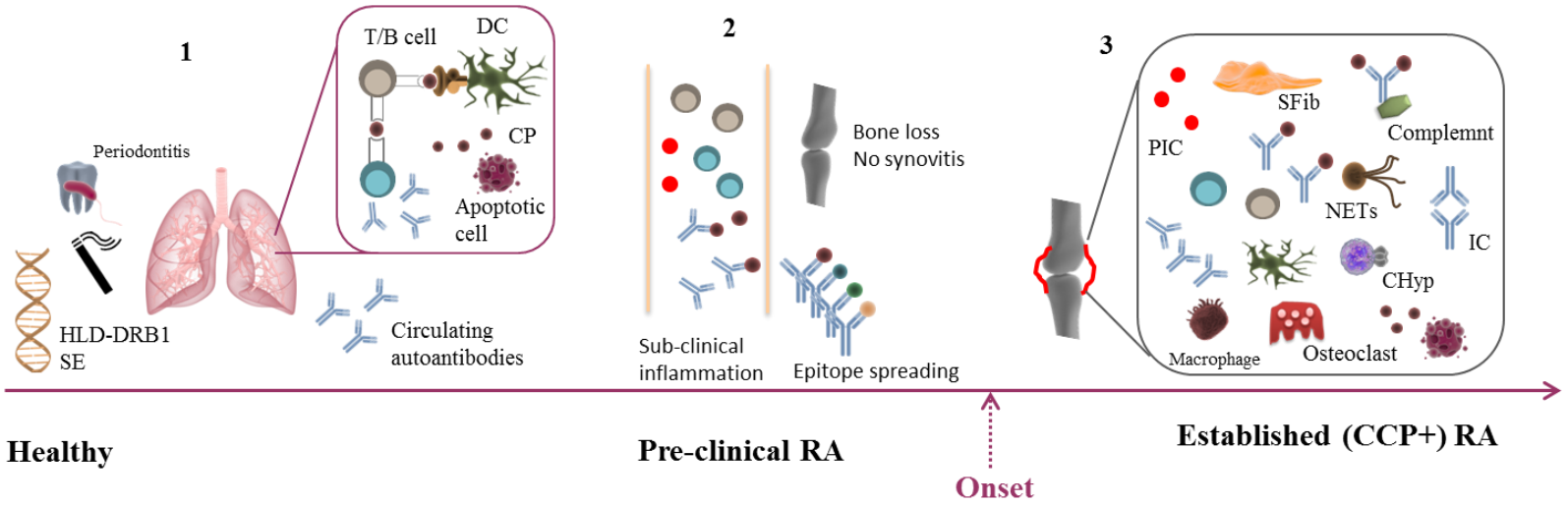


Figure 5 Hypothesis for the initiation and development of CCP/ACPA+RA. RA is a multifactorial disease whereby an interaction between a certain genetic background and different environmental risk factors may trigger an initial extra-articular (e.g. lung) immune response that ultimately reaches the synovial joints. **(1)** Circulating ACPAs (as well as other autoantibodies) are detected before signs of inflammation in the synovial joint; ACPA+ individuals who are smokers and carry the HLA-DRB1 SE genes display an increased risk of developing RA in comparison to the ACPA-RA population. Smoking leads to an increased number of citrullinated proteins in the lungs. Apoptotic cells leak PAD enzymes into the extracellular environment and additional proteins become citrullinated (as the concentration of calcium is higher extracellularly). An enrichment of ACPA as well as presence of ectopic lymphoid structures (in untreated ACPA+RA and ACPA+ individuals without joint involvement) has been reported in the lungs. Citrullinated proteins are processed and presented by DC to T cells through HLA SE molecules, which leads to activation of B cells and production of autoantibodies. **(2)** Pre-clinical RA is characterized by sub-clinical systemic inflammation visualized by magnetic resonance imaging (MRI) but no clinical signs of synovitis. In line, MRI of non-RA ACPA+ individuals show no inflammation in the joints. However, bone loss has been reported in ACPA+ subjects before RA diagnosis, suggesting that bone may be affected prior to the synovial membrane. Close to the clinical onset ACPA undergo epitope spreading and a simultaneous rise in circulating pro-inflammatory cytokines and chemokines takes place. **(3)** A second hit (trauma or infection) may be necessary for the autoimmune response to reach the synovial joints. ACPA are enriched locally in the SF (with higher prevalence of certain reactivities such as α-enolase), bind to local citrullinated proteins and mediate effector functions that drive and perpetuate synovial inflammation and bone loss: activation of complement (via both the classical and alternative pathways); generation of TNF-α by macrophages; induction of osteoclastogenesis and bone loss; activation of synovial mast cells through TLR engagement; stimulation of macrophages through engagement of both TLR-4 and FcγR. ACPAs were recently shown to associate with NETs, which are an additional source of citrullinated proteins. Perforin and membrane attack complex-medicated hypercitrullination occurs in SF cells ("cellular hypercitrullination"); Citrulline reactive-B cells are also abundant in the SF and may function as APC. The presence of common citrullinated proteins in the lung and synovial joint adds to the lung/joint axis hypothesis of RA pathogenesis. Periodontitis may also be an additional driver for the pathogenesis of RA. Antibodies targeting the bacteria *Porphyromonas Gingivalis* (PG) have been detected in RA patients and in individuals at risk. An association between periodontitis and RA has also been reported. Furthermore, PG express its own PAD raising the possibility that bacterial PAD may citrullinate proteins other than the ones with bacterial origin. *SE, shared epitope; DC, dendritic cell; CP, citrullinated proteins; IC, immunecomplexes; PIC, pro-inflammatory cytokines; SFib, synovial fibroblasts; NETs, nuclear extracellular traps. CHyp, Cellular hypercitrullination.*

ACPA IgG, IgM and IgA isotypes have all been documented in RA patients, and they appear to have distinct distribution in comparison to healthy relatives (19% of the unnafected relatives of those RA patients we ACPA+) (209). Within ACPA IgG, IgG1 is the most common form and is present both in circulation and in the SF of RA patients. Notably, ACPA IgG was detected in BAL from early RA and sputum from individuals at risk of developing RA (Figure 3) (83, 201, 209). Interestingly, the expansion of ACPA isotype distribution occurs before the onset of RA, and similarly to what is observed for epitope spreading, it does not magnify after the diagnosis (181, 191). Similarly to ACPA IgG, also circulating IgA isotype was found before RA diagnosis. In this study, both the isotypes were found to predict the development of RA (210). In fact, the number of isotypes was found to correlate with RA development and progression and further, the isotype profile at disease onset predicted radiographic damage (211). Nonetheless, ACPA IgA was recently found in saliva of RA patients that develop less severe joint erosion, which is suggestive of a mucosal ACPA IgA-mediated anti-inflammatory function (212).

The **affinity** of antibodies develops overtime. In a primary immune response the affinity is generally low, increasing throughout the course of the response. This is called affinity maturation and the process is enhanced by lower levels of antigen. As mentioned above, avidity provides a more accurate assessment of the binding strength between an antibody and its cognate antigen. ACPAs were shown to have low avidity in comparison to antibodies against recall antigens (tetanus toxoid and diptheria toxoid) and to undergo affinity maturation prior to onset of arthritis, without further maturation after diagnosis. In addition, patients with ACPA displaying lower avidity presented more joint destruction than those with higher avidity. These findings may suggest that low-affinity ACPA are more effective at binding citrullinated antigens. In line, the constant supply of citrullinated antigens may lead to a continuous production of ACPA thus, perpetuating the pathogenic immune response (213-215). The joint erosions associated with ACPA+ disease may be due to the effector functions low avidity ACPA execute, e.g. more effective at activating the complement system (213). In paper IV we explore the possible existence of high affinity ACPA subtypes among the total RA ACPA pool.

The **glycosylation patterns** of an antibody have a major influence on its functionality since they affect the physiochemical properties of the antibody by altering its structure and conformation. These alterations have implications, for example, in the bond between IgGs and Fcγ receptors (FcγR) or complement components as well as in antibody dependent cell-mediated toxicity. Interference with antigen recognition due to glycosylation of the antibody has also been reported. Antibodies may regulate the immune response in a pro- or anti-inflammatory way depending on the glycosylation features. Antibodies containing sialic acid residues in the constant fragment display a more anti-inflammatory profile (216).
In serum, the Fc part of ACPA was shown to be more agalactosylated and to lack sialic acid residues, in comparison to the non-ACPA IgG1-Fc part (217, 218). In addition, ACPA existing in SF differs from ACPA circulating in serum and, furthermore, ACPA-glycans were also shown to vary among the four isotypes (IgG1-4) (217, 219). ACPA Fab-peptide sequences seem to differ from non-ACPA RA IgG. Very recently, Rombout *et al* (220) showed that ACPA Fc glycans exhibit pro-inflammatory properties already before the disease onset. Furthermore, hyperglycosylation of ACPA variable domains was reported, whereas no

such alterations were registered for other autoantibodies or antibodies against recall-antigens (221). Together, these findings provide evidences for the importance ACPA glycosylation patterns have in mediating pro-inflammatory responses and thus, ACPA pathogenicity.

5.5. Pain in RA

Pain is one of the most disabling features of RA. A large majority of patients consider pain treatment the factor that needs better improvement in the disease management (222). Although different treatments are given to RA patients, non-steroidal anti-inflammatory drugs (NSAIDS), non-biological disease-modifying anti-rheumatic drugs (DMARDs) or biologics, the pain often persists after decrease of joint inflammation, improvement of disease activity or even when patients are in remission. Additionally, arthralgia (joint pain), in many cases, develops before synovitis and is predictive of development of RA (223-225).

Pain results from the perception and recognition of an injured organ that has been exposed to noxious thermal, mechanical or chemical stimuli (nociception). The nerve cells that primarily sense and respond to these stimuli by sending signals to the spinal cord and brain are called nociceptors. Anatomically, the cell bodies of nociceptors locate both in the dorsal root ganglia (responsible for the body area) and in the trigeminal ganglia (responsible for the face). In addition, nociceptors have a peripheral branch that innervates the organs, and a central branch that connects with the spinal cord. Dorsal root ganglia (DRG) and trigeminal ganglia (TG) are groups of neurons responsible for conveying sensory information from the periphery to the central nervous system (226-228).

Nociceptors are high-threshold fibers that can broadly be divided into categories depending on axonal size and thus conduction speed. Another way to classify nerves is according to their response to environmental stimuli: mechanical, thermal or chemical. The nociceptors that sense and transmit acute and fast pain (retraction behaviour) contain myelinated afferent A delta fibers (Aδ) of medium diameter (type I). These Aδ fibers can be sub-classified in two additional types: one responds to mechanical and chemical stimuli while having a high threshold for heat, and the other type senses thermal stimuli while having a higher threshold for mechanical stimulation. Additionally, type I nociceptors comprise Aβ fibers of a larger diameter that are responsible for conducting mechanical stimulation. Type II nociceptors comprise unmyelinated fibers of small diameter (C fibers) that perceive slow pain. Also here fibers respond to both mechanical and thermal stimuli. In an inflammatory context these fibers lower their thresholds and become increasingly sensitized to otherwise normal stimuli (226-228).

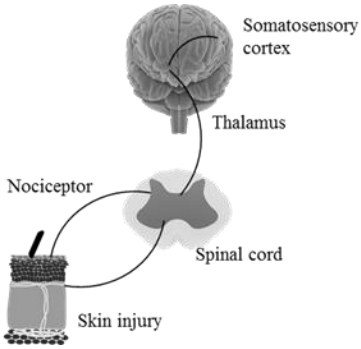


Figure 6 Pain follows a route that starts with the painful sensory stimuli being conducted by the afferent nociceptors to projecting neurons on the spinal cord. From here the information about the location and intensity of the stimulus reaches the somatosensory cortex of the brain via thalamus.

The information conducted by the nociceptors after detecting a nociceptive stimuli (thermal, mechanical or chemical), is detected by specific receptors such as thermo-sensitive channels and mechano-transduction channels that are expressed by the nerve fibers. Voltage-gated ion channels are then activated in order to generate action potentials so the signals can reach the spinal cord and from there get to the brain where the pain is perceived (226-229).

Persistent or chronic pain

Similarly to the immune system, pain normally exerts an important protective role in the body. However, when persistent nociceptive input is dysregulated it can lead to hypersensitivity and pain signals become chronic and disabling. Processes such as allodynia (an innocuous stimuli is perceived as painful) or hyperalgesia (when the painful stimuli provokes more intense pain) become long-lasting and do not resolve as normally would happen. This is often the case for individuals suffering from RA and other inflammatory rheumatic disorders. Both central and peripheral mechanisms are responsible for the persistence of pain in these diseases (226-231).

Peripheral pain mechanisms in RA are normally mediated by inflammatory factors **(inflammatory pain)**. At the site of injury, a whole set of inflammatory molecules (released by nociceptors and non-neuronal cells) and cells accumulate. Macrophages, mast cells, neutrophils, platelets, fibroblasts and endothelial cells secrete and release pro-inflammatory and pro-algesic factors such as neurotransmitters, eicosanoids (prostaglandins, thromboxane, and leukotrienes), cytokines, chemokines and bradykinin. The nociceptors become activated since they contain receptors that bind the pro-inflammatory and pro-algesic factors, which lead to increased hypersensitivity to the injury and sometimes to persistent pain (226, 231, 232).

At the **central level**, the hypersensitivity that leads to chronic pain is mediated by processes that involve the central nervous system **(central sensitization)** (226, 231, 233).
Some examples are:

(1) Modifications in the receptor neurotransmission, for example glutamate/N-methyl-D-aspartate (NMDA). When there is a persistent painful stimulus, nociceptors release glutamate and other neurotransmitters in the dorsal horn of the spinal cord. NMDA receptors that are usually at rest become activated and induce a signaling cascade that facilitates the transmission of pain to the brain.
(2) Dysregulation of central pain modulatory pathways. Disinhibition if pathways involve GABAergic (gamma-aminobutyric acid) and Glycinergic neurotransmitters. When a persistent injury occurs, the neurotransmitters GABA and Glycine lose their inhibitory capacity and the regulation of pain is impaired.
(3) Glial (microglia and astrocytes)-neuronal interactions. In a setting of persistent nociceptive input, a cascade of events activates microglial cells. These cells will thereafter secrete cytokines (e.g. TNF-α, IL-1β and IL-6) that will further enhance pain signals in the dorsal horn (229, 234).

With the exception of cartilage, the sensory nerves (nociceptors) locate in all structures of the joint and surrounding areas (ligaments, tendon sheaths, muscles, adipose tissue and bone). These nociceptors are composed of Aδ, Aβ and C type of fibers and thus, both thermal and

mechanical stimuli are conducted from the skin and deep areas (joint and nearby regions) to the spinal cord. In addition, the nociceptors communicate with neurons located in supraspinal structures, and in this case only signals from the deep areas are conducted (235, 236).
The gap between pain and inflammation in RA demonstrates that several molecular mechanisms are responsible for inducing pain in RA (236-239). Paper II of the thesis explores the role of ACPA as potential pain mediators.

6. Idiopathic inflammatory myopathies

Idiopathic inflammatory myopathies (IIM), collectively called myositis, encompass a group of chronic inflammatory diseases mainly affecting the skeletal muscle. Muscle weakness and low endurance as well as inflammatory cell infiltrates in muscle tissues are common manifestations. However, organs such as lungs, skin, joints, heart and gastrointestinal tract are also frequently involved (240, 241). A common clinical feature among patients affected with IIM is the difficulty of performing tasks that involve the proximal muscles. Daily activities, such as climbing stairs, getting dressed, lifting objects, bathing and combing the hair become hard to execute. Neck-flexor and pharyngeal muscles may be affected, leading to difficulties swallowing (dysphagia). The heterogeneous phenotypic nature of muscle biopsy features, autoantibodies presence and differential organ involvement, suggest different possible etiologies and distinct operating molecular pathways. These systemic diseases are classically termed as polymyositis (PM), dermatomyositis (DM), necrotizing myopathy (NM) and inclusion-body myositis (IBM) (68, 69, 242, 243).

A concerted interaction between environmental factors and genetically susceptible individuals has been proposed as the main initial trigger of IIM. Evidences of such interaction lie, for instance, in the association between anti-Jo1 autoantibodies, HLA-DRB1*03 and smoking (57). In addition, a link between statins exposure and development of anti-HMGCR antibodies was shown for individuals carrying HLA DRB1*11:01 alleles (244). A viral aetiology has also been suggested, although no conclusive proofs are yet available. Different myopathies were reported to be associated with viral infections namely, human immunodeficiency virus, human T-cell lymphotropic virus type I, coxsackie virus cytomegalovirus, and Epstein-Barr virus (65-67, 245-248).

Several molecular mechanisms, immune-mediated and non-immune mediated, may drive muscle damage (249, 250). In this thesis, immune-mediated mechanisms will be addressed and further discussed in the following sections. Emphasis will be given to the intricate and fascinating HisRS antigen and its cognate autoantibodies.

IIM has a prevalence of 8.7-19/100,000 individuals and an incidence of 8-13/1 000 000 person-years (251-254). DM and PM affect more women than men whereas IBM is more dominant in men. No distinction is seen in NM regarding gender. The age of onset can be, in the case of DM, during childhood or later in adulthood; PM on the other hand is more prevalent in adulthood as is the case for NM, although elderly individuals are also affected by NM; IBM tends to develop mainly in elderly over 50 years old (241, 255-257).

6.1 Classification and diagnostic criteria – clinical features and laboratory findings

Several classification and diagnostic criteria have been proposed for IIM. However, the Bohan and Peter criteria are the most commonly used in clinical practice for the classification of PM and DM (Table 5a) (242, 243). The diagnostic criteria for IBM were proposed in 1995 by Griggs and colleagues and are listed in Table 5b (258).

Table 5a Diagnostic and classification criteria postulated by Bohan and Peter in 1975. The described criteria provide basis for the diagnosis and classification of DM and PM.

Criterion	Description
1	Symmetrical muscle weakness (usually progressive) of limb-girdle and neck flexors
2	Muscle biopsy: Necrosis of type I and II muscle fibers; phagocytosis, regeneration, variation in fiber size, perifascicular atrophy and inflammation
3	Elevated serum levels of muscle-associated enzymes (creatine kinase, aldolase and transaminases)
4	Electromyographic abnormalities: myopathic potentials, short and low-amplitude polyphasic action potentials; Fibrillation potentials; high-frequency repetitive charges
5	Cutaneous manifestations, concordant with myositis (heliotrope rash or Gottron's papules)
Definitive PM – Four criteria are fulfilled and no skin involvement; **Definitive DM** – Four criteria are fulfilled, together with skin rash. **Probable PM and possible PM** require fulfilment of three and two criterion without skin involvement, respectively. **Probable DM and possible DM** are diagnosed when two criteria and one criterion, together with skin rash, are presented.	

Table 5b Diagnostic and classification criteria postulated by Griggs and co-authors in 1995. The described criteria provide basis for the diagnosis and classification of IBM.

Criterion	Description
A. Clinical features	1. Duration of disease >6 months 2. Age of onset >30 years old 3. Muscle weakness - The proximal and distal muscles of arms and legs must be affected. In addition, the patients must show ≥1 of the following trades: i) Finger flexor weakness; ii) Wrist flexor weakness more serious than wrist extensor weakness; iii) Quadriceps muscle weakness
B. Laboratory features	1. Serum creatinine < 12 times normal 2. Muscle biopsy: i) inflammatory myopathy with mononuclear cell infiltrates of nonnecrotic fibers; ii) vacuolated muscle fiber; iii) intracellular amyloid deposits or 15-18 nm tubulofilaments 3. Electromyography must be consistent with features of inflammatory myopathies
C. Family history	Rarely IBM is observed in families. If there is a case, inflammation must be observed in the muscle biopsy
Definitive IBM – Patients must exhibit all muscle biopsy characteristic features, irrespective of other clinical or laboratory findings. **Possible IBM** – The muscle only exhibits inflammation (and not the other typical muscle biopsy characteristics) and the patients fulfil A1, 2 and 3 and B1 and 3.	

Among other limitations, these criteria do not take into consideration the occurrence of autoantibodies. Up to 80% of IIM patients have autoantibodies in circulation (69, 259, 260). Some of these autoantibodies are not specific for myositis (**myositis associated autoantibodies, MAA**) and may even translate an overlap of several connective tissue disorders (CTD), e.g. PM or DM with scleroderma, SLE, Sjögren's syndrome or RA (36, 261). MAA are autoantibodies that target the autoantigens PM-Scl, TRIM21, U1-RNP

(RNA-binding domain on the peptide component of U1 ribonucleoprotein complex), and Ku (nuclear heterodimer composed of 70 and 80 kDa subunits of DNA binding protein).
Autoantibodies directed to HisRS (anti-Jo1 or anti-HisRS) or other aminoacyl transfer RNA synthetases (anti-aaRS), anti-HMGCR, anti-MDA5 and anti-TIF1γ autoantibodies are designated **myositis specific antibodies** (**MSA**) and are more specific for IIM (Table 6) (260, 262-264). Evidence has been gathered clearly showing an association between certain MSA and distinct clinical phenotypes (e.g. anti-aaRS antibodies and anti-synthetase syndrome, ASS)(69, 260, 262). Love *et al* (265) proposed a different method to classify IIM patients whereby the presence of MSA was taken into account. In addition, the association among autoantibodies, different symptoms and genetic background was also investigated. The stratification based on occurrence of MSA created a more defined and homogenous classification in comparison to categorization into the classical clinical groups (e.g. PM, DM and IBM). The authors concluded that to have information on MSA status would benefit the interpretation of clinical symptoms, prognosis and clinical course. The diagnostic and prognostic value of MSA is currently well accepted. Table 6 lists MSA and associated clinical phenotypes. In the specific case of MSA that target aaRS, I will dedicate a separate section (6.2.1).

Table 6 Myositis specific antibodies (MSA) and related clinical phenotypes (264).

Autoantibody	Clinical phenotype
Anti-MDA5, Melanoma differentiation-associated protein 5	Amyopathic DM; ILD with poor prognosis
Anti-SRP, Signal recognition particle	Necrotizing myopathy
Anti-TIF1-γ, Transcriptional intermediary factor 1 γ	Severe DM; Cancer-associated DM; Juvenile DM
Anti-Mi-2, Nucleosome remodeling deacetylase complex	Mild DM
Anti-HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase	Necrotizing myopathy (NM)
Anti-SAE, Small ubiquitin-like modifier activating enzyme	DM
Anti-NXP-2, Nuclear matrix protein 2	Severe DM; cancer-associated DM

6.1.1 Dermatomyositis (DM)

DM patients primarily exhibit skin manifestations such as heliotrope rash with edema (violet rash and swelling of eyelids) and Gottron's papules (red slightly elevated rash over the knuckles). Additional skin manifestations may also be present and may sometimes overlap with other CTD: rash on the face, knees, elbows, neck, chest and shoulders. Overall, these symptoms may be accompanied or preceded by muscle weakness. The muscle biopsy in typical cases shows signs of inflammation (complement deposition, $CD4^+$T cells, B cells and pDC infiltrates and MHC-I over-expression) in the layer of connective tissue that maintains the muscle fibers clustered (perimysium), around the vessels (perivascular) and around the bundles of fibers (perifascicular). Atrophy, necrosis and regeneration of the fibers may be seen particularly with a perifascicular localization (240, 241, 266, 267). Dermatomyositis may be associated with cancer. Up to 32% of DM patients were reported to develop cancer within 3 to 5 years after DM diagnosis (240, 268, 269).

6.1.2 Polymyositis (PM)

PM is many times diagnosed by exclusion of other myopathies. Similarly to DM, patients show proximal symmetric weakness, overlap with other CTD and presence of autoantibodies, myalgia and arthralgia. Muscle biopsy typically reveals inflammatory cells localized to the endomysium (connective tissue that surrounds each muscle fiber) with muscle fibers expressing MHC-I, surrounded by $CD8^+$ T cells, macrophages, myeloid DCs and less frequently plasma cells, and no vacuoles in the fibers. Degeneration or regeneration of muscle fibers is also common in PM but with a scattered pattern in the fascicle (240, 241, 257).

6.1.3 Inclusion body myositis (IBM)

IBM develops gradually with early involvement of both proximal and distal muscles and early muscle atrophy. It may also be associated with a CTD. Muscle biopsy features are similar to PM with addition of rimmed vacuoles in the muscle fibers and with inclusions in the nuclei or cytoplasm. Autoantibodies are less common in IBM but may be present in up to 20% of the patients, often anti-Ro60/52 (270). A new IBM associated antibody, anti-cytosolic 5'-nucleotidase 1A, has recently been discovered to occur in 33-76% of IBM patients (271, 272). Very recently these autoantibodies were also demonstrated in 15% of DM, 23% of Sjögren's syndrome and 14% SLE patients (273). If IBM is a primary inflammatory myopathy such as DM and PM, is still under debate (274, 275).

6.1.4 Necrotizing myopathy (NM)

NM associates with malignancy, CTD or statin use. Patients have muscle weakness and autoantibodies directed against HMGCR or signal-recognition particle (SRP). Muscle fiber necrosis and deposits of complement components in the capillaries portrait NM muscle biopsy. Inflammatory cells are scarce or absent (240, 241, 257).

6.2 Anti-synthetase syndrome (ASS)

The clinical entity characterized by the simultaneous presence of anti-aaRS autoantibodies, inflammatory myopathy, ILD, arthritis, fever, Raynaud’s phenomenon (RP, limited blood supply to areas such as fingers and toes) and mechanic’s hands is termed anti-synthetase syndrome (ASS, Figure 7, upper panel) (276). To date, eight aaRS have been documented as autoantigenic targets, and therefore eight correspondent autoantibodies were described: Anti-histidyl-RS or aJo1, the most common; anti-threonyl-RS or aPL-7; anti-alanyl-RS or aPL-12; anti-glycyl-RS or aEJ; anti-isoleucyl-RS or aOJ; anti-asparaginyl-RS or aKS; anti-tyrosyl-RS or aHa and anti-phenylalanyl-RS or aZo (Figure 7, lower panel).

From the clinical features that characterize ASS, one of the most frequent symptoms, also contributing to high morbidity and mortality, is ILD (277, 278). It has been reported that between 67-100% of ASS patients are affected by ILD (279-282). Some authors claim that this percentage depends on the anti-aaRS antibody specificity. In fact, evidence suggests an association between the presence of certain anti-aaRS and the clinical presentation of ILD, which may suggest different sub-phenotypes within ASS (279, 281, 283-285). For example, aPL-7 appears to be more associated with muscle weakness and heliotrope rash, whereas aJo1 has been correlated with ILD, arthritis, mechanic hands and muscle weakness. Anti-PL-12

was described to preferentially link to RP and isolated ILD; Anti-KS and aOJ have also been associated with isolated ILD (285-288). Intriguingly, anti-aaRS antibodies rarely overlap in the same individual. Histopathologically, ASS is characterized by regeneration, fragmentation and inflammation (macrophages and lymphocytes infiltrates) of the perimysial and perifascicular areas as well as increased protein expression of MHC-I (Figure 7) (289-291). Similar histological findings can be observed either when myositis is diagnosed alone or in the case of ASS diagnosis.

Although ASS has been considered an individual clinical entity, a recent meta-analysis demonstrates a large overlap between clinical features of anti-aaRS-positive patients and those having anti-Pm-Scl autoantibodies. Based on their findings, the authors question the validity of ASS diagnosed as an entity (292).

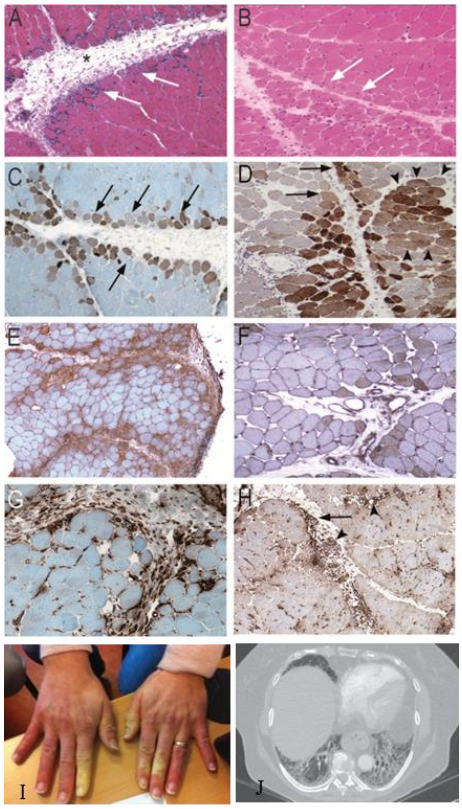


Figure 7 Myopathology of ASS (left panel) and anti-aaRS autoantibodies (Table below). Illustrative examples of ASS (pictures A, C, E, G) and DM (pictures B, D, F and H) histopathology. **A**, Regenerating fibers and infiltrates in perimysium and perifascicular endomysium; **B**, Perifascicular atrophy; Neural cell adhesion molecule expressing-myofibers in the perifascicular layers in **C** and in individual or grouped perifascicular layers in **D**; MHC-I expressing fibers in **E** (more pronounced) and **F**; **G** and **H**, Macrophages precursor (CD68) expression mainly in the perimysium; **I**, Raynaud's phenomenon in an aPL7 positive patient; **J**, High-resolution computed tomography from the lungs of an aJo1+ patient.

Images A – H were reprinted with permission from BioMed Central, Aouizerate J et al. Acta Neuropathologica Communications 2014, 2:154. Images I and J were reprinted with permission from @ERS publications, Jean-Christophe Lega et al. European Respiratory Review Jun 2015, 24 (136) 216-238; DOI: 10.1183/16000617.00002015.

Autoantibody	Frequency in IIM (%)	References
Anti-histidyl RS (aJo1)	15-36	(285, 293, 294)
Anti-threonyl RS (aPL-7)	2-18	(285, 295-297)
Anti-alanyl RS (aPL-12)	2-11	(285, 297-299)
Anti-glycyl RS (aEJ)	2-23	(285, 297, 300)
Anti-isoleucyl RA (aOJ)	<5	(285, 287, 301, 302)
Anti-asparaginyl RS (aKS)	<8	(285, 303, 304)
Anti-tyrosyl RS (aHA)	<1	(305)
Anti-phenylalanyl RS (aZo)	<1	(306)

6.2.1 The case for HisRS and anti-HisRS antibodies

Histidyl-tRNA synthetase (HisRS or Jo1) is an important enzyme for protein synthesis. HisRS catalyses the attachment of histidine (standard amino acid) to its cognate transfer RNA ($tRNA^{His}$). The human HisRS gene locates on chromosome five, and four main isoforms are

produced by alternative splicing. Several natural variants (a mutation or a polymorphism) have also been documented. For instance, mutations located at positions 454 and 137 were found in patients with Usher syndrome 3B (progressive hearing and visual loss during childhood) and peripheral neuropathy (distal motor and sensory dysfunction) (307, 308).

Higher eucaryotic HisRS, as well as other aaRS, were appended with an additional domain not comprised earlier in the phylogenetic tree. This domain, termed WHEP domain, is composed of 60 amino acids and locates in the N-terminal. The main human isoform studied (and also used in this thesis) is composed of 509 amino acid residues; it has a molecular weight of 57.4 kDa, and exists as a homodimer (~115 kDa). Natural splice variants (SV) have been reported and are indicative of an evolutionary pressure to perform additional non-canonical functions (309, 310). Other aaRS were also described to have alternative fragments and to be implicated in functions other than protein synthesis (311). HisRS structure is depicted in Figure 8.

Antibodies directed to HisRS (aJo1) are the most common type of MSA (Figure 7, table in lower panel). Most remarkable is the association with ILD whereby up to 90% of the patients with aJo1 in circulation present signs of ILD (279, 312). Whether the autoantibodies are involved in disease initiation and development is still an open question. Yet, several observations suggest a potential role: aJo1 serum levels correlate with disease activity and may precede the clinical symptoms (313-315); HLA-DRB1*03 genotype is associated with smoking and the presence of aJo1 (57); aJo1-positive sera induces IFN-α production by pDCs (270); In a conditional MHC-I animal model, mice developed myositis–like disease, and clinical features such as aJo1 were observed (316). Furthermore, MHC-I as well as type I IFN-induced genes are highly expressed in muscle fibers from myositis patients in comparison to normal muscle (317, 318). Knowing that type I IFN are strong MHC-I inducers (319), it is tempting to suggest that one mechanism by which aJo1 may mediate pathogenesis is via IFN (further discussed in results and discussion section).

Anti-Jo1 autoantibodies bind both HisRS as well as the complex HisRS-$tRNA^{His}$, and display higher affinity to HisRS if the enzyme has been pre-incubated with tRNA thus, acting as an inhibitor of HisRS enzymatic activity (315, 320). A truncated version of HisRS lacking the amino acids 1-60 was revealed enzymatically inactive and no longer antigenic (321). These experiments were crucial to understand that one of aJo1 main epitopes is located near the N-terminal. It was also shown that aJo1 from IIM patients recognize more than one epitope within HisRS (both conformational dependent and indepedent), suggesting that the antibodies undergo epitope spreading and may not result from a cross-reactive antigen (e.g. virus) (315, 322). Different myositis patients recognize different HisRS epitopes (322). In addition, aJo1 were shown to be mainly IgG1 isotype and to undergo affinity maturation and class switching (323). Taken together, these studies indicate that aJo1 may have a relevant role in IIM (313, 315, 320-324).

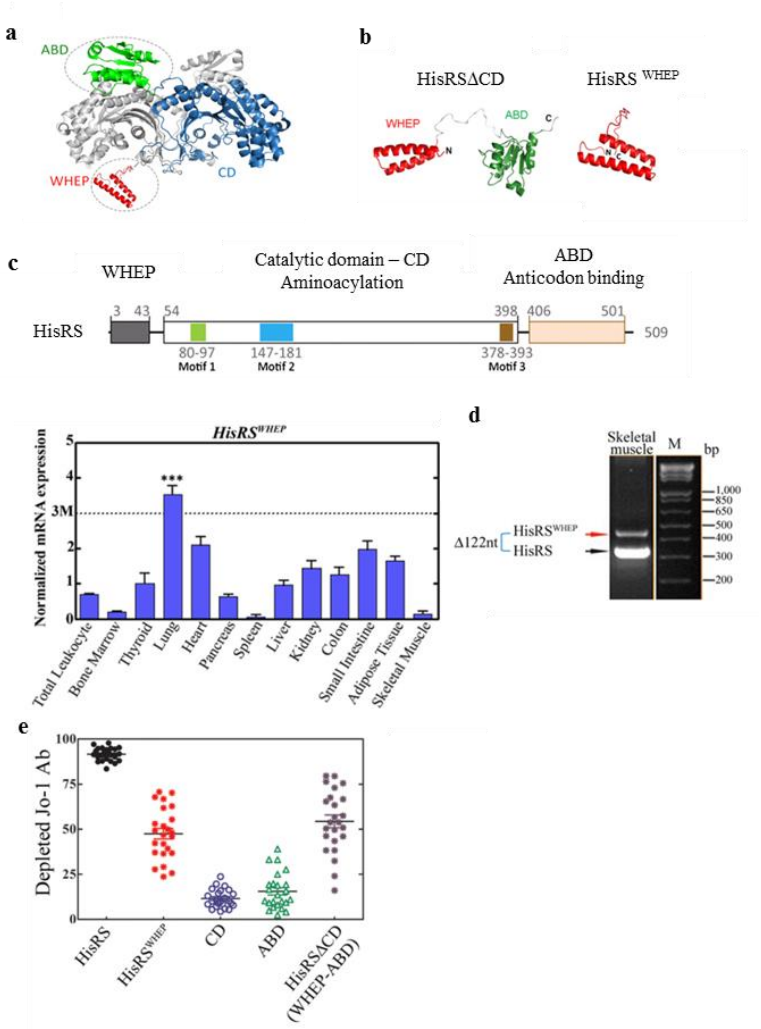


Figure 8 HisRS structure. a and **c** HisRS is composed of three main domains: N-terminal WHEP domain (comprises the first 60 amino acids), a core catalytic domain (CD) and a C-terminal anticodon binding domain (ABD); **b** A natural splice variant (SV) lacking the entire CD, thus joining the N-terminal WHEP to the C-terminal ABD domains, was first described in 2012 and termed HisRSΔCD. HisRSΔCD exists as a 171 amino acid monomer. An additional and also previously undescribed SV is the WHEP domain, solely composed of the first 60 amino acid ($HisRS^{WHEP}$); **c** Motifs 1, 2 and 3 (colored green, blue and brown, respectively) represent conserved sequences; **d left panel**, lung from healthy donors and **d right panel**, skeletal muscle from myositis patients express messenger RNA that encodes for $HisRS^{WHEP}$ SV; **e** Anti-Jo1+-depleted serum recognizes HisRSΔCD and $HisRS^{WHEP}$ SV whereas minimal reactivity was observed for CD and ABD. The authors claimed that the two SV contain the major anti-Jo1 epitopes. *Figure 8 contains sections from Figure 1 and 2 published by Zhou JJ et al. J Biol Chem. 2014 Jul 11;289(28):19269-75 (Images available from JBC publications).*

Albeit no conclusive proof of the pathogenic involvement of aJo1 in initiation and development of IIM with lung involvement, HisRS, on the other hand, has been suggested to drive tissue specific autoimmunity and damage. In fact, one may speculate whether the immune system would employ aJo1 as an attempt to neutralize HisRS damages. Figure 9 illustrates a summary of the current lines of investigation whereby HisRS is shown to be a crucial player that drives lung and muscle autoimmunity (325-327). The work done in this thesis will add some more pieces to the understanding of autoimmunity in IIM with lung involvement (Paper V). Our findings are described under sud-heading 4 of the section Results and Discussion and illustrated in Figure 17 of Conclusions and Future Perspectives section.

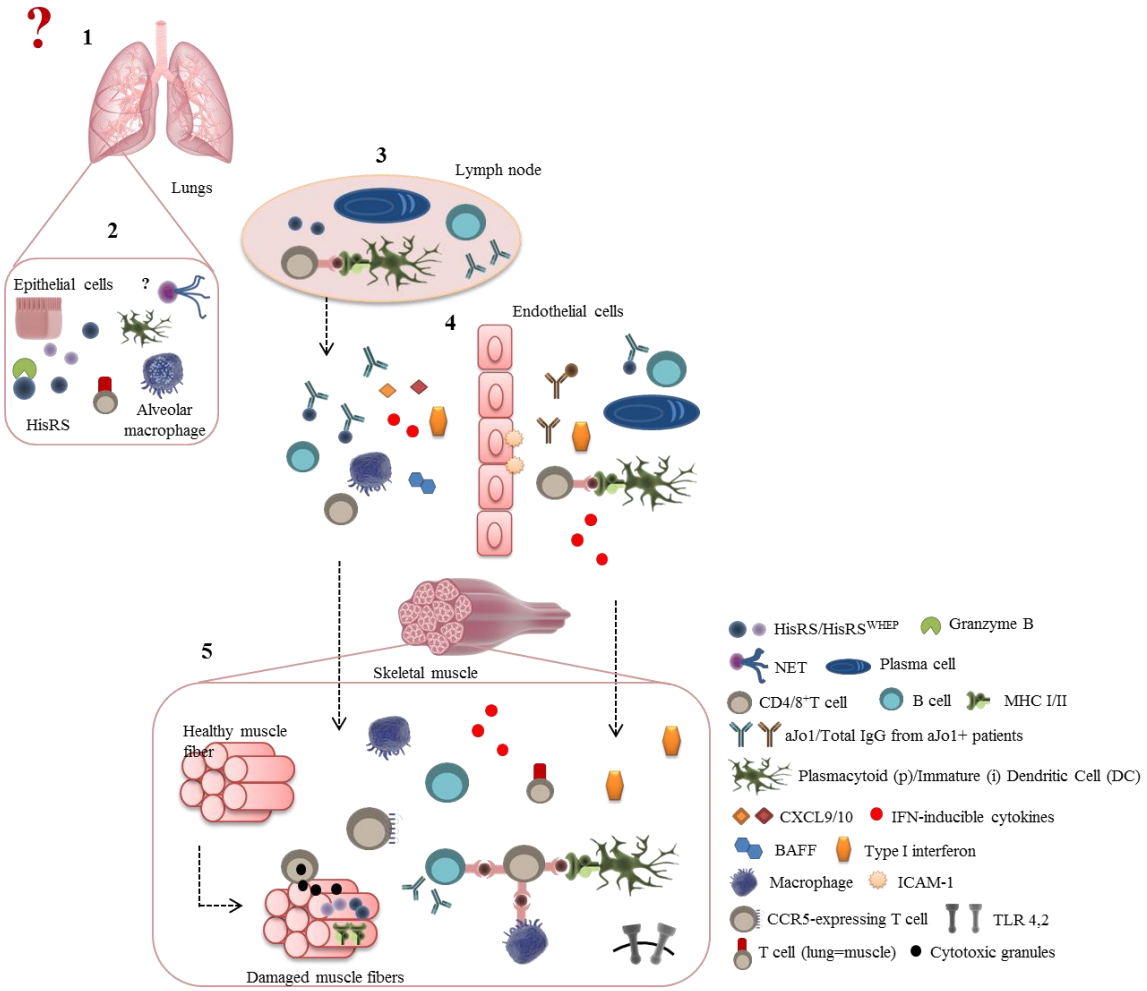


Figure 9 Insights into HisRS-mediated mechanisms – impact on IIM with lung involvement. The hypothesis of HisRS involvement in IIM with lung involvement starts with a question mark **(1)**. After years of research the trigger(s) leading to IIM remain unidentified. Environmental factors such as smoking or viral infections may be the lead word, though not yet conclusive. **(2)** In certain individuals, when such trigger is present and together with a particular genetic background (e.g. HLA-DRB1*03 (57)), HisRS is cleaved by GrB released by $CD8^+$T cells in the lungs. The GrB cleavage site in HisRS is located close to the main epitope, near the N-terminal (328). In addition, a HisRS SV containing the first 60 amino acids of the protein ($HisRS^{WHEP}$) is also expressed in high amounts in the lung (310), suggesting that in this tissue HisRS is antigenic and amenable to initiate a local autoimmune response. As local iDC and macrophages take up HisRS, they become activated and migrate to the lymph nodes **(3)** to present HisRS peptides to T cells. T and B cells are activated, proliferate, undergo clonal expansion and release pro-inflammatory cytokines. As B cells differentiate into plasma cells, aJo1 are produced and travel to the blood stream **(4)**. Naïve T and B cells will also reach the target organs. Sera from aJo1+ individuals induce expression of intercellular adhesion molecule-1 (ICAM-1) (329) which in turn may foster the inflammatory flux namely, activated T cells that will penetrate the endothelial cells and reach peripheral lymphoid tissues **(4)**. Type I IFN seems to have an important role in HisRS-mediated autoimmunity. Sera or total IgGs from aJo1+ sera induce increased IFN production by peripheral blood mononuclear cells (270). Anti-Jo1+ patients with ILD have high levels of B cell activating factor (BAFF), which is known to be a potent IFN inducer (330). In line, aJo1+ patients with ILD show IFN-γ-inducible chemokine (CXCL9 and CXCL10) patterns, distinct from those observed in anti-SRP+ myositis patients with no ILD (279). **(5)** Muscles, the other target microenvironment, from myositis patients undergo regeneration in order to replace injured fibers, leading to over-expression of HisRS (98). HisRS displays pro-inflammatory properties by activating CCR5-T lymphocytes and iDC, thereby inducing inflammatory cell recruitment to the damaged muscle (99). As damaged fibers are being replaced constantly, the over-expression of HisRS directs the immune response against the regenerating fibers thus creating a feed-forward loop that leads to additional HisRS protein expression and ultimately to muscle injury **(5)**. $CD4^+$ and $CD8^+$T cell infiltrates release cytotoxic granules (containing perforin

and granzymes) that cause fiber necrosis. HisRS was described to mediate TLR2 and 4 activation which suggests an intrinsic capacity to induce myositis in an adaptive-immunity independent manner (331, 332). Macrophages, DC, pDC and T and B lymphocytes mount a local immune response that perpetuates inflammation and tissue damage **(5)**. A possible link between the lung and the muscle in myositis is the T cell receptor usage, shown to be shared between the two organs (333) **(2, 5)**. IIM patients with ILD were described to promote defective development of NETs, which may be an additional explanatory mechanism for the development of lung disease in these patients (334) **(2)**.

7. Therapies

7.1. Rheumatoid arthritis

The spectra of treatments used for RA have significantly developed in the last decades. Due to an advanced knowledge on molecular mechanisms, more targeted therapies have been proposed and some of them have already being implemented.

By definition, **synthetic DMARDs** (disease modifying anti-rheumatic drugs) need to have an impact on inflammation and reduce joint damage (reduce acute-phase proteins and autoantibody levels and delay erosions, evaluated by radiological assessment) (335, 336). Methotrexate (MTX) and glucocorticoids are two of the most common DMARDs used in clinical practice. *Glucocorticoids* (GluC) are steroid hormones that effectively suppress synovial joint inflammation by acting at various molecular levels like inhibition of cytokine transcription or induction of apoptosis in inflammatory cells (337). Although effective, GluCs cause serious side-effects (338-340). *Methotrexate* is an anti-metabolite that inhibits enzymes responsible for the nucleic acids and protein synthesis, therefore a powerful modulator of the cell metabolism. It is well tolerated and acts effectively limiting symptomology and relieving radiographic progression. However, gastrointestinal problems and pulmonary interstitial lung disease are potential serious side-effects (338, 340, 341). **Biological DMARDs** (Table 7) have gain importance in the past decade as RA molecular mechanisms became unraveled. These biological agents mimic endogenous components such as antibodies and receptors.

Table 7 Biological agents used for the treatment of RA

Biological agent	Structure	Target
Infliximab	Human chimeric monoclonal antibody	TNF
Adalimumab	Fully human monoclonal antibody	TNF
Etanercept	Recombinant TNF receptor fused to a human Fc protein	TNF
Golimumab	Human monoclonal IgG1	TNF
Certolizumab pegol	Recombinant Fab' conjugated to polyethylene glycol	TNF
Anakinra	Recombinant IL-1 receptor antagonist	IL-1 receptor
Tocilizumab	Human receptor antibody	IL-6 receptor
Abatacept	Fc region from IgG1 fused to CTLA-4	T cell modulation
Rituximab	Chimeric monoclonal antibody anti-CD20	B cell depletion

Five TNF inhibitors are currently in use (340, 342-344). Treatment with TNF inhibitors successfully decreases synovitis and bone destruction, thereby preventing physical disability. Furthermore, improved clinical efficacy has been shown when combined therapy is administered (e.g. MTX + TNF inhibitors). Other biological agents (Table 7) have proven beneficial treating inflammation and bone destruction (345-347). Although promising therapies, a fraction of patients treated with biologicals do not respond at all and some develop complications such as infections (338, 348).

Pain

In order to reduce pain, RA patients are often given non-steroidal anti-inflammatory drugs (NSAID, e.g. aspirin and ibuprofen). The mechanism of action involves inhibition of cyclooxygenase (COX)-1 and 2, as these enzymes are responsible for the synthesis of prostaglandins. Administration of DMARDs is also used for pain management in RA. However, and as described in the section above, pain does not only result from inflammation and damaged tissue, therefore alternative and/or complement analgesics are in need (225, 236).

7.2. Idiopathic inflammatory myopathies

Because IIM are rare conditions clinical trials are difficult to design due to the low prevalence and incidence and heterogeneous clinical presentation. Additionally, the lack of molecular knowledge responsible for disease mechanisms limits the design of more personalized drugs. Therapy in IIM aims to improve muscle strength, prevent disease progression and reduce additional organ damage. The first line treatment for PM and DM include high doses of GluCs (e.g. prednisone) for several weeks followed by slow tapering (349, 350). Often immunossuppressive agents such as azathioprine and MTX are given in combination to GluCs (241, 257, 351, 352). When this approach fails either due to side-effects or low improvement of physical disability, additional immune-modulating agents are used. Those agents consist of cyclosporine, mycophenolate mofetil, tacrolimus and intravenous immunoglobulins (350, 353, 354). All these compounds interfere with proliferating T and B cells. Azathioprine is metabolized through a number of steps that ultimately inhibit *de novo* synthesis of purines and consequently DNA (355), mycophenolate mofetil inhibits inosine-5'-monophosphate dehydrogenase which is an enzyme responsible for purine synthesis (356) and tacrolimus inhibits a calcium-dependent protein important for IL-2 signaling (357). Biological agents have also been used in myositis although, so far, with unclear or no effects (349, 350).

Increasing number of evidences demonstrate the benefit of adding physical exercise to the pharmacological treatment. Exercise appears to contribute to improved muscle performance through various mechanisms: up-regulation of genes involved in muscle growth and aerobic pathways; down-regulation of inflammation-related genes; alterations in the type of muscle fibers; and enlargement of muscle fiber area (358-361).

7.3. A non-conventional approach – Targeting autoantibodies for disease amelioration

Albeit autoimmune conditions share a dysregulation of the immune system, distinct molecular mechanisms account for the disease development. Different genetic backgrounds, environmental exposures or other stochastic factors dictate the outcome phenotype of these disorders. The development of personalized therapies has been possible due to a rise in the molecular knowledge behind the classification of sub-populations of patients such as for example, ACPA+ and ACPA- RA or RF+ and RF- RA. Rituximab was reported to be more efficient on seropositive RA (ACPA+ and RF+) in comparison to the seronegative population (ACPA- and RF-) (346, 362, 363). Although rituximab is appreciated as an effective B-cell depleting agent leading to disease progression arrest, naïve B cells were demonstrated to persist, re-populate and induce relapse (364). An attractive alternative approach is the direct target of pathogenic autoantibodies by small molecules such as peptides or nucleotides (365).

Such principle has been explored in conditions such as cardiomyopathy whereby myocardial damage is known to be caused by autoantibodies towards cardiac β1-adrenergic receptor (366). In the case of RA, endogenously citrullinated peptides could be engineered into stable compounds. The binding of these compounds to ACPA would restrict perpetuation of inflammation and therefore improve disease outcome (365, 367). This strategy was explored in paper III and IV of the thesis and will be further discussed in results and discussion section.

AIMS

Inflammatory autoimmune diseases are chronic disabling conditions that affect organs such as joints, muscles, lungs and skin. In order to treat or prevent these diseases we need a better understanding of the contributory pathogenic events. Hence, the overall aims of this thesis were to provide insights into different molecular mechanisms underlying rheumatoid arthritis and myositis as well as to develop compounds that counteract the effects of autoantibodies by blocking the binding to their cognate targets.

Specific aims

Paper I: Development of a new methodology for ACPA purification from plasma and synovial fluid in order to use the antibodies as a molecular tool to investigate their mode of action.

Paper II: Investigate if purified human ACPA induce pain in mice.

Paper III: Evaluate if endogenously citrullinated fibrinogen peptides are autoantigenic and whether those peptides could be utilized to block human ACPA.

Paper IV: Development of new stable compounds intended to specifically block citrullinated fibrinogen-reactive ACPA and therefore be employed as a therapy and as a diagnostic tool.

Paper V: Investigate the contribution of the antigen HisRS and cognate autoantibodies in myositis with lung involvement.

EXPERIMENTAL PROCEDURES

1. Patients (Papers I-V)

Samples from patients that visited the Rheumatology Clinic at Karolinska University Hospital (Stockholm, Sweden) and fulfilling the ACR/EULAR criteria for RA (122-124) were collected. Selection of patients for isolation of anti-CCP2 IgG was based on anti-CCP2 positive reactivity. Plasma (n=62), sera (n=10) and synovial fluid (SF, n=34) from the selected, non-paired RA patients, were collected and kept at −80°C until processed. Synovial fluid was taken from patients that underwent arthrocentesis (procedure executed with a needle in order to aspirate fluid from a joint). The purified autoantibodies were employed in several experiments that compose papers I to IV. For Paper III, sera from 927 (402 CCP2 positive and 525 CCP2 negative) newly diagnosed RA patients (within one year of appearance of first symptoms), were used. Simultaneously, 461 healthy controls (HC) sera from the Swedish population registry were randomly chosen in order to match age, sex, and residential area. All samples were taken within the context of the Epidemiological Investigation of RA (EIRA) case-control cohort.

In paper V, sera from myositis (PM, DM and IBM) patients (aJo1+ (n=38) and aJo1- (n=15)) were collected in the Rheumatology Clinic at Karolinska University Hospital. Paired BAL-serum samples were collected from myositis, sarcoidosis and HC (n=7-8 per group) in the Respiratory Medicine Unit at Karolinska University Hospital. The bronchoscopy was performed according to published procedure (368). Presence or absence of anti-Jo1 antibodies was assessed by Elisa and/or Line-blot and/or WB/IP assays (369, 370). Satellite cells for *in vitro* experiments were derived from the muscle biopsy of a DM patient, as described in Pandya *et al* (under revision). Myositis patients fulfilled the Bohan and Peter criteria for diagnosis of PM and DM, and Griggs criteria for diagnosis of IBM. Criteria designed by the World Association of Sarcoidosis and other Granulomatous Disorders (WASOG) (371) was applied for sarcoidosis.

2. *In vitro* methodology

2.1. Peptides (Papers III and IV)

The following peptides were synthetized in house:

Table 8 Sequences of linear peptides utilized in paper III

Peptide name	Peptide sequence
Arg573	HHP GIA EFP S**R**G KSS SYS KQF
Cit573	HHP GIA EFP S**X**G KSS SYS KQF
Arg591	SKQ FTS STS YN**R** GDS TFE SKS
Cit591	SKQ FTS STS YN**X** GDS TFE SKS
Arg72,74	APP PIS GGG Y**R**A **R**PA KAA AT
Cit72	APP PIS GGG Y**X**A RPA KAA AT
Cit74	APP PIS GGG YRA **X**PA KAA AT

R – Arginine; X – Citrulline.

For the sake of clarity the nomenclature of peptides in paper III was altered. Table 9 lists all the peptides and nomenclatures used in paper III and IV.

Table 9 Nomenclature of peptides utilized in paper III and IV

Peptide name paper III	Peptide name paper IV
Cit573	[Cit573]fib(563-583)
Arg573	[Arg573]fib(563-583)
Cit573Lin1	[Cit573]fib(566-580)
Cit573Lin2	[Cit573]fib(567-577)
Cit573Cyc	c[Cit573]fib(563-583)
Peptides only included in paper IV	
c[Cit573]fib(566-580)	
c[Arg573]fib(566-580)	
c[Ala573]fib(566-580)	
s1[Cit573]fib(566-580)	
s1[Arg573]fib(566-580)	
s2[Cit573,Arg575]fib(566-580)	
s3[Cit573,Arg575]fib(566-580)	

Cit, citrulline; Fib, fibrinogen; Arg, arginine; Lin1 – Linear form 1, which is the truncated Cit573 peptide with 15 amino acids residues; Lin2 – Linear form 2, which is the truncated Cit573 peptide with 11 amino acids residues; Cyc – cyclic; C, cyclic; Ala, alanine; s, sunflower trypsin inhibitor 1 (SFTI-1); s2 and s3 are mutant peptides of s1.

S1[Cit573]fib(566-580) peptide was synthetized by grafting the truncated linear peptide [Cit573]fib(566-580) into the stable cyclic peptide sunflower trypsin inhibitor-1 (SFTI-1). S2 and s3 are mutants of s1 in which a Lys was inserted in different parts of the secondary loop of SFTI-1. In addition, the Lys in the position 575 was exchanged to an Arg in order to avoid unspecific binding. S2[Cit573,Arg575]fib(566-580) was used to create an affinity column (methods 2.3) and to develop an in house ELISA (methods 2.4.2).

2.2. Expression and purification of recombinant human HisRS (Paper V)

Recombinant human HisRS (rHisRS) was overexpressed in *Escherichia coli* (*E. coli*) BL21(DE3)pLysS competent cells. Recombinant HisRS was purified from the cytosolic fraction of bacteria lysate by sub-cellular fractionation followed by hypdroxyapatite and strong anion exchange chromatography. Thereafter, rHisRS was simultaneously filtered and buffer exchanged to PBS using 30 kDa filter tubes. Purity was assessed by SDS-PAGE followed by Coomassie blue and silver staining as well as by WB using a commercial primary antibody (Anti-HisRS N-terminal). Mass spectrometry analysis confirmed the recombinant protein as human HisRS. No endotoxin contamination was found (<0.05 EU/mL).

2.3. Affinity purification of autoantibodies from plasma, sera and SF (Papers I – V)

The development of a methodology for isolation of autoantibodies from patients' material (blood and SF) was one of the most important techniques used throughout my PhD studies (purification scheme is illustrated in Figure 13, under sub-heading 1 of results and discussion section). These autoantibodies were utilized in all papers and manuscripts included in the thesis and are continuously used in additional studies, not addressed herein. The main core of this methodology was applied for the purification of anti-CCP2 IgG (also termed ACPA), anti-citrullinated fibrinogen α chain derived peptide [Cit573]fib(563-583) IgG (anti-Cit573 IgG) and anti-Jo1 IgG, and includes two main steps: isolation of total IgG followed by isolation of specific autoantibody fine-specificities utilizing in house prepared affinity columns (CCP2, s2[Cit573,Arg575]fib(566-580), and rHisRS columns). To prepare

s2[Cit573,Arg575]fib(566-580) and HisRS affinity columns one mg of either s2[Cit573,Arg575]fib(566-580) peptide or rHisRS were coupled to 1 mL N-hydroxysuccinimide activated pre-packed sepharose columns.

Synovial fluid samples were treated with hyaluronidase, centrifuged at 3000 g during 5 minutes and precipitated with saturated ammonium sulfate. Before applying the samples to HiTrap Protein G HP columns, SF-derived proteins were dialyzed against PBS and plasma and sera were centrifuged at 3000 g for 5 minutes and diluted 1:5 (v/v) in PBS. As total IgGs were eluted with 0.1 M glycine-HCl pH 2.7 the pH was immediately adjusted to ~7.4 (1 M Tris, pH 9) and the antibodies were dialysed against PBS. Anti-CCP2 IgG, anti-Cit573 IgG and anti-Jo1 IgG were isolated by applying total IgGs to CCP2, s2[Cit573,Arg575]fib(566-580) or HisRS affinity columns and further processed as described for total IgGs. Non-CCP2, non-s2[Cit573,Arg575]fib(566-580) and non-HisRS-reactive IgGs were collected to use as controls (Flow Through, FT). Recovery, purity and specificity of eluted autoantibodies were confirmed by Coomassie blue staining, WB/dot-blot or enzyme linked immunosorbent assay (ELISA) after antibodies have been buffer exchanged to PBS and sterile filtered. The concentration and proportion (%) of anti-CCP2 IgG, anti-Cit573 IgG and anti-Jo1 IgG in plasma/sera or SF was estimated by measuring the absorbance at 280 or 595 nm, taking into consideration the amount of total IgG loaded into each affinity column and the amount of eluted antibodies.

Throughout the thesis anti-citrullinated protein/peptide antibodies affinity purified according to the method described above will be called either ACPA or anti(a)-CCP2 IgG. Several ACPA/aCCP2 IgG pools were prepared and utilized in Paper I-IV.

2.4. ELISA (Papers I, III, IV and V)

The ELISA is a method vastly used both for research purposes as well as diagnostics in the clinical practice. This technique enables the detection and quantification of proteins in a complex matrix such as sera or plasma. The principle of an ELISA is based on the reaction between antigen and antibody. This type of interaction is also widely applied by different methodologies such as WB, dot-blot and IP, as discussed in the following sections. During my PhD studies I performed commercial ELISAs such as the anti-CCP2 ELISA and previously developed in-house ELISAs such as anti-Cit573 ELISA. Also, I established new ELISA assays for detection of anti-Cit573 IgG using the new compound s2[Cit573,Arg575]fib(566-580), detection of anti-HisRS IgG, IgA and IgM as well as a sandwich ELISA to detect and quantify HisRS antigen in plasma/sera, BAL and supernatants from myoblasts and myotubes. Cutoff values for ELISA were calculated as the average OD plus two times the standard deviation (SD) calculated from the OD given by the control group.

2.4.1. Competition assays (Paper III and IV)

Competition experiments were developed in order to assess ACPA inhibition by different citrullinated fibrinogen-derived peptides. Peptides derived from the fibrinogen α (Arg/Cit573, Arg/Cit591) and β chains (Arg/Cit72, Arg/Cit74), previously identified in the synovial tissue from RA patients (Table 8 (372)) were tested in the assay. Figure 10 illustrates the competition experiment developed.

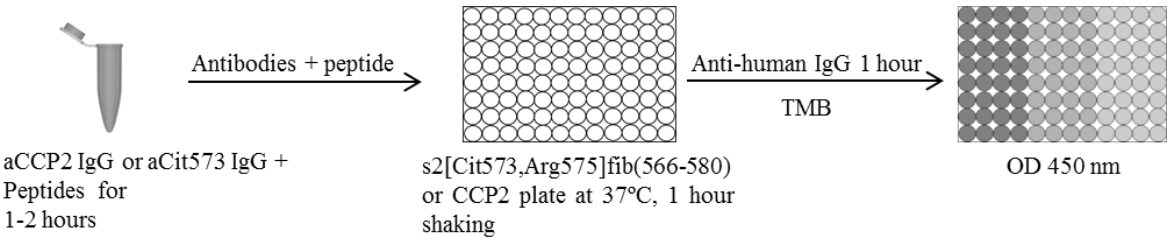


Figure 10 Serial increasing amounts of each peptide (0.016 - 320 nmol in 225 µl of dilution buffer, citrulline, arginine and alanine versions) were incubated for one to two hours with two different affinity purified anti-CCP2 IgG pools (8 nM). Autoantibody pools comprising aCCP2 IgG antibodies were isolated from sera and plasma samples collected from RA patients (Methods 2.2). Antibodies mixed with peptides were subsequently added to CCP2 or s2[Cit573,Arg575]fib(566-580) plates. After incubation with anti-human IgG antibody, absorbance at 450 nm was recorded. Percentage (%) of ACPA inhibition was calculated according to the following formula:

ACPA inhibition (%) = 100-[(OD_{450nm} ACPA + peptide)/OD_{450nm} ACPA with no peptide]*100,

considering ACPA incubation with no peptide as 100% of maximum OD_{450nm} obtained.
Additionally, aCit573 IgG isolated from three RA individuals (Methods 2.3) known to contain this specific ACPA reactivity were also tested in the competition assay.

Truncated, cyclic and grafted versions of the linear peptide [Cit573]fib(563-583) were also tested (Structures and terminology are listed in Tables 8 and 9, Methods 2.1 and in Figure 14 under sub-heading 3 of Results and Conclusions).

2.4.2. Anti-Cit573 IgG ELISA using a novel compound (Paper IV)

In order to ensure that s2[Cit573,Arg575]fib(566-580) would bind to 96 well plates through the N-terminal, Nunc® Immobilizer™ Amino plates were chosen. Plates were coated with s2[Cit573,Arg575]fib(566-580) (5 µg/mL peptide diluted in 100 mM Na_2CO_3 pH 9.6) overnight at 4°C, washed three times with PBS containing 0.05% Tween, and blocked for 60 minutes with PBS/1% BSA. Diluted plasma (1:100) was added to blocked plates for one to two hours at room temperature followed by incubation with anti-human IgG antibody (1:10,000, one hour at room temperature). The substrate 3,3',5,5'-Tetramethylbenzidine was added to washed plates and the enzymatic reaction was stopped with 1 M H_2SO_4. Absorbance at 450 nm (reference filter 650 nm) was recorded.

2.4.3. Anti-Jo1 Igs ELISA (Paper V)

Anti-Jo1 Ig isotypes were investigated both in BAL and sera from myositis patients and HC similarly to what was described above for anti-Cit573 IgG (Section 2.4.2). For detection and quantification of aJo1 Igs (IgG, IgA and IgM), sera was diluted 5000x for aJo1 IgG, 890x for aJo1 IgA and 500x for anti-Jo1 IgM ELISAs. Secondary antibodies were diluted 500x and 50x for detection of human IgG/IgA and IgM, respectively. Absorbance at 405 nm (p-nitrophenyl phosphate substrate) was recorded between 15 and 45 minutes and compared to absorbance given by total Igs ELISA. Anti-histidyl tRNA synthetase autoantibodies will be denoted anti(a)-Jo1 IgG, aJo1 or anti-HisRS IgG throughout the thesis.

2.5. Western blot and dot-blot (Paper I, II and V)

Western blot (WB) is an analytical technique, which allows identification and semi-quantification of specific proteins in complex protein mixtures. During my PhD studies I

have widely applied WB to various purposes. In Paper II the localization of human antibodies (anti-CCP2 IgG; CCP2 FT IgG and HC IgG) that have been injected in mice was investigated in homogenized mice tissues and plasma using a single antibody that targets human IgG. In Paper V, WBs were performed in plasma, sera and BAL from myositis patients, sarcoidosis patients and HC and also in eluates obtained by immunoprecipitation of plasma, sera and BAL from the same individuals in order to investigate the presence of extracellular HisRS. Figure 11 illustrates the WB procedure.

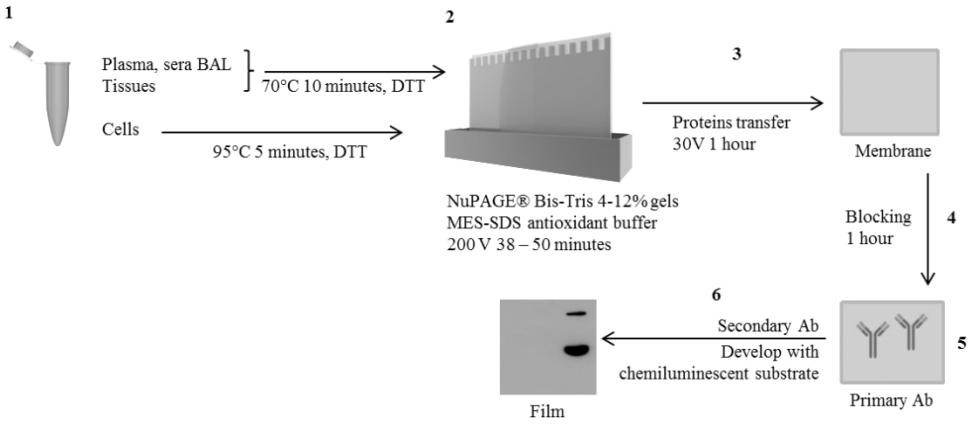


Figure 11 Sample preparation **(1)** for WB requires different approaches depending if working with fluids (sera, plasma or BAL), tissues or cells. Tissues and cells were first treated (homogenized and sonicated) with protein extraction buffer supplemented with protease inhibitors. Extracted proteins were reduced and denatured and separated using electrophoresis according to their molecular weights **(2)**. Subsequently, proteins were electroblotted from the gel onto a membrane (nitrocellulose or polyvinylidene fluoride) **(3)** and non-specific binding of the membranes was blocked with 5% non-fat dry milk in PBS or TBS containing 0.1% Tween 20 **(4)**. Incubation with the antibody targeting the protein of interest (Primary Ab) generally took place overnight at 4°C **(5)**. In the case of WB in Paper II, membranes were incubated with anti-human IgG for one hour at RT, washed and directly developed as described below. The last step involves the incubation of the protein-containing membranes with the secondary antibody (one hour at RT, **6**). This antibody is chosen based on the reactivity against the animal where the primary antibody was raised in. Lastly, membranes were developed and images acquired using X-ray films. Native gels required non-reducing/denaturing gels and buffers (NativePAGE Novex 4-16% Bis-Tris) but the remainder protocol follows the descriptions above.

Dot-Blot

Dot-blot is a simplified version of WB. One advantage of using dot-blot is the fact that low abundant proteins present in complexes matrixes such as sera and plasma can readily be seen in the membranes, whereas sometimes no signal can be detected on WB (on both native and denaturing/reducing conditions). This method was applied in Paper V: for dot-blot experiments plasma, sera or BAL were directly spotted to nitrocellulose membranes, let dry for ~15 minutes, incubated with blocking buffer and the following steps follow those described above for WB.

2.6. Stability assay (Paper IV)

Stability of SFTI-1 scaffolded peptides and related peptides (Table 9) was evaluated both in human sera and whole-blood. Sera, blood or PBS were incubated at 37 ºC with 200-250 µM of each peptide (incubation with blood samples was always performed with gently shaking in order to avoid coagulation) at selected time-points (t=0, 10, 60, 180, 330 and 1400 minutes). At the end of each time-point, 6 M urea was added to the samples and incubated for 10 minutes at 5°C. After adding 20% trichloroacetic acid (TCA), samples were centrifuged at

13,000 g for 10 minutes. Supernatants were collected and analyzed by liquid chromatography-mass spectrometry (Shimadzu LC-10 HPLC system connected to a Thermo-Finnigan LCQ Deca electrospray ion trap MS). Run was done at 0.3 mL/minute for 30 minutes using a linear gradient of 5% to 90% acetonitrile in 0.05% formic acid. In order to calculate the percentage of remaining peptide, the area under the curve of the peak of each peptide at each given time-point of incubation with sera, blood or PBS was calculated and compared to the amount of the same peptide at the initial time (t=0).

2.7. Immunoprecipitation (Paper V)

Immunoprecipitation is a technique employed for isolation and detection of proteins using a specific antibody that recognizes a particular antigen. It enables the purification of non-abundant proteins in complex matrixes such as sera or plasma therefore, enhancing the concentration of the protein target in comparison to the initial sample which facilitates the identification. It also enables the study of PTMs and protein-protein interactions. Due to the fact that we could not detect HisRS by WB but only using dot-blot, and as a matter of confirmation, an IP method was developed. Moreover, we have evidence that a minimum of 10 ng of HisRS is necessary to be able to visualize the protein when analyzing sera/plasma by WB (at least utilizing the methodology available in our laboratory). Considering that not only the WB, but also the ELISA ran for HisRS detection in sera/plasma and the recombinant protein itself has being troublesome, we concluded that the 3D structure of the protein is somewhat sensitive and when bound to a structure such as 96 well plate, it may no longer be available for visualization (e.g. due to steric hindrance). We anticipate that differential centrifugation of plasma into microparticles and exosomes will provide better visualization of the protein (discussed in Conclusions and Future Perspectives section).

Immunoprecipitation of HisRS from sera samples was undertaken using protein G-coupled magnetic sepharose beads that have been incubated with 1 μg/mL commercial anti-HisRS-C-terminal antibody for 20 minutes. Beads were cross-linked with 50 mM DMP (dimethyl pimelimidate dihydrochloride) in 200 mM triethanolamine at pH 8.9, blocked with 100 mM ethanolamine pH 8.9 and incubated overnight at 4°C with 10 μL sera diluted in 100 μL TBS pH 7.5. Subsequently to several steps of non-harsh wash, proteins were eluted with 4% acetic acid and detection was executed by WB using anti-HisRS-C and anti-HisRS N-terminal as detection antibodies.

2.8. Cell culture of myoblasts and myotubes (Paper V)

In-vitro grown myotubes (derived from myoblasts) are a good representative of adult myocytes and can be used as a laboratory tool to study, for instance, the effect of autoantibodies or certain drugs in the local inflammatory milieu, fibers contractility and muscle weakness related pathways. The study of muscle cell proliferation and differentiation is also possible recurring to *in-vitro* grown myotubes.

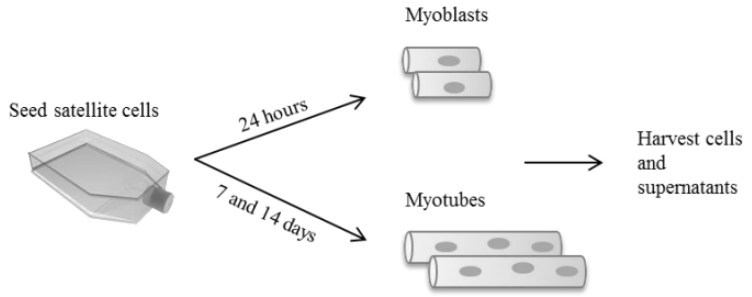


Figure 12 Satellite cells (previously prepared from a muscle biopsy, as described in Pandya *et al* 2015, under revision) were expanded in collagen type I-coated flasks, using DMEM GlutaMax/20% FBS, penicillin, streptomycin and fungizone containing media. For the myoblasts proliferation it is important to use cell culture media containing >10% FBS. When confluent, fresh DMEM GlutaMax/20% FBS containing media was added and the myoblasts were harvested 24 hours later. In a parallel experiment, myoblasts were cultivated in DMEM GlutaMax containing only 2% FBS so they could fuse and form myotubes.

2.9. Platelet-related experiments (Paper V)

Platelets experiments were performed in order to investigate HisRS unknown non-canonical functions (311). Platelet rich plasma (PRP) was obtained from healthy donors (n=4) blood, collected in sodium citrate-containing vacutainer tubes and centrifuged at 150 g for 11 minutes. Serial concentrations of rHisRS (0.05 to 200 pM) or ADP (positive control, 1 to 8.6 µM) were incubate with PRP for 15 minutes at RT. Thereafter, PRP-rHisRS/ADP mixture was incubated with CD62P (P-selectin) antibody for 20 minutes in darkness. Platelet activation was assessed by measuring P-selectin expression by flow cytometry.

3 *In vivo* model of pain (Paper II)

In order to investigate the impact of ACPA in the development of pain that frequently precedes joint inflammation in RA patients, affinity purified ACPA (anti-CCP2 IgG, Paper I) were injected intravenously in male B10.RIII and BALB/c mice. Animals were also systemically injected with monoclonal murinized ACPA antibodies. Further, and with the intent of understanding the molecular mechanism responsible for ACPA-induced pain, mice were given intra-articular (ankle joint) injections (isoflurane anesthesia was used when performing the injections thereof) of CXCL1, CXCL2 (30 ng each or 30 ng in a 1:1 mixture). Additionally, mice were injected subcutaneously with CXCR1/2 antagonist (reparixin), twice a day, 30 mg/kg/day. After the injections, mice were monitored for mechanical hypersensitivity, which can be assessed by calculating the threshold amount of force required to withdraw the paw from a given applied stimulus. In order to do that, von Frey filaments (373) were used to apply the stimuli in the hind paw and the withdrawal thresholds were estimated using Dixon up-down method (374). Heat and cold sensitivity were assessed as follows: heat, mice were placed in a temperature controlled container where a radiant thermal stimulus is applied from under the glass where the mice are standing (modified Hargreaves box (375)); cold, a drop of acetone was applied to the hind paw. After applying the thermal stimuli, sensitivity was assessed by measuring the duration of nocifensive behaviour, such as lifting, shaking, biting, and licking the paw. Another feature of pain was assessed by recording the locomotor activity via monitoring the night activity of the animals, utilizing Oxymax/Comprehensive Lab Monitoring System equipped with infrared sensors for movement detection in three axes (X, Y and Z). Total movement, ambulation and rearing were determined. On the day of sacrifice, organs were collected for later determination of inflammation markers and fate of injected antibodies.

4 Statistics

In paper I, Mann-Whitney U test for independent groups was employed in order to determine the differences in antibody levels among both the ACPA reactivities in plasma and SF as well as in the anti-CCP2 IgGs eluted from the CCP2 column. In paper II, the following statistical models were used: Two-way ANOVA to analyze changes over-time and one-way ANOVA to address differences in > three groups, both followed by Bonferroni post-hoc test; differences between two groups were investigated using Student's t-test; for arthritis and histological scores the Kruskal-Wallis test was used followed by Dunn's multiple comparison post hoc test. In paper III and IV, comparison between competition percentages performed by the different ACPA inhibitors was investigated using Student's t-test. Cutoff values for microarray chip data (ISAC system) were calculated as the 98th percentile reactivity among the EIRA controls included in the study. All statistical analysis, including calculations of IC_{50} values, were performed using GraphPad Prism 6 software. IC_{50} was considered as defined by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification: 'the molar concentration of an antagonist that reduces the response to an agonist by 50%' (376). In Paper V, Spearman nonparametric function was used to search for correlations between autoantibodies in BAL and sera (the confidence interval assumed was set to 95%). $P<0.05$ was assumed to denote a significant difference.

RESULTS AND DISCUSSION

1. Affinity purified anti-citrullinated protein/peptide antibodies target antigens expressed in the rheumatic joint (Paper I)

An increased number of studies demonstrate the pathogenic potential of ACPA (97, 194, 199, 377-379). In study I we developed a new methodology to purify human ACPA from plasma and synovial fluid (SF) from patients with RA. The isolation of these autoantibodies was intended to acquire unique molecular tools for future studies on the pathobiology of ACPA.

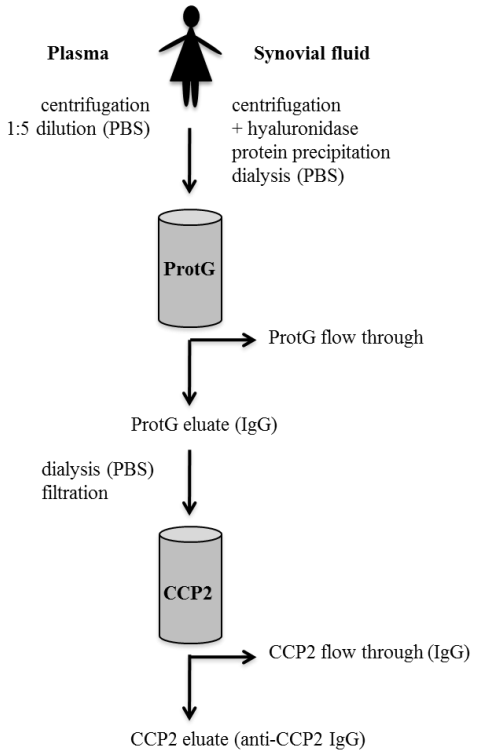


Figure 13 Schematic illustration of the anti-CCP2 IgG purification process

Using the procedure illustrated in Figure 13 we efficiently purified anti-CCP2 IgG from both plasma and SF. By first applying the samples on protein G columns we isolated total IgGs that were afterwards efficiently separated in two fractions, CCP2 eluate (anti-CCP2 IgG) and CCP2 flow through (non-CCP2 reactive IgG, FT), using a CCP2-affinity column. This methodology provided high recovery of pure, intact and reactive anti-CCP2 IgG. Additionally, it enabled the measurement of the protein content of all chromatography fractions and thus, a more accurate estimation of the concentration and proportion of anti-CCP2 IgG in both plasma and SF. Although the median concentration of anti-CCP2 IgG was more elevated (0.2 mg/ml) in plasma in comparison to SF (0.06 mg/ml), the proportion of CCP2-reative IgG was in fact slightly higher in SF (2.2% compared to 1.5% in plasma). These observations are in line with a recent study whereby a similar technique was utilized to isolate anti-CCP2 reactive IgG, and are likewise concordant with other antigen-specific IgG responses (380-382). Further, SF from four patients revealed a percentage of CCP2-reactive IgG above 6% of the total IgG pool, whereas in plasma the highest sample registered presented < 4% CCP2-reactive IgG molecules. This result suggests a local enrichment and perhaps also local production of ACPA in the synovial rheumatic joint, an observation in line with a previous work that showed increased titers of ACPA in SF (192), and further sustained by a recent study where the authors demonstrated that ~25% of the synovial IgG-expressing B cells are CCP2-reactive (76).

Anti-CCP2 IgG collected from the CCP2 column were analysed by in-house peptide ELISAs and were demonstrated to contain ACPA of different fine specificities. Namely, CCP2 eluates isolated from both plasma and SF reacted against α-enolase (CEP-1; amino acid residues 5-21), vimentin (Cit-vim; amino acid residues 60-75), fibrinogen α chain (Cit-fib; amino acid residues 36-52 and 563-583, the former was not included in the article), and collagen type II (Cit-C1; amino acid residues 359-369), demonstrating that CCP2 can act as a surrogate marker for different ACPA reactivities. Our data confirms and provides additional evidence to a previous study that showed the possibility of eluting fibrinogen-reactive ACPA from CCP2 ELISA plates (383). The different composition of ACPA reactivities may account for

the distinct CCP2 binding profiles observed for each patient sample after ELISA analysis, although the CCP2 eluates were ran at the same concentration. However, no association was observed between a specific ACPA reactivity and the CCP2-binding. Interestingly, some anti-CCP2 IgG (especially those purified from the SF) presented simultaneous high CEP-1 reactivity and weaker CCP2-binding. Similar observations were reported by another study in which reactive B-cells were described to be more commonly found in SF (76). Although this finding may suggest that anti-CEP-1 IgG have low anti-CCP2 affinity, particularly in SF, it may simply mean a better CEP-1 ELISA performance, in comparison to the other peptide ELISAs. Nevertheless, the CCP2 column efficiently bound all anti-CEP-1 IgGs since no reactivity was found in the control FT. The reactivity of CCP2 eluates to different citrullinated proteins was confirmed by WB. In addition, we could also demonstrate that anti-CCP2 IgG bind *in-vivo* generated citrullinated proteins.

To conclude, in Paper I we developed an efficient method to purify anti-CCP2 IgG from RA plasma and SF thus providing molecular tools that can be employed to study ACPA-mediated immune responses. This methodology enabled an accurate measurement of CCP2-reactive IgG in both fluids and further provided evidence for the use of CCP2 as a surrogate marker for several ACPA reactivities.

2. Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism (Paper II)

Joint inflammation is frequently preceded by arthralgia (joint pain) and patients in remission continue to suffer from mild, moderate or severe pain (223, 224). ACPA is often detected before clinical onset of RA and predicts development of arthritis in patients with arthralgia (70, 384). In paper II we addressed whether ACPA were able to induce pain in mice, independent of joint inflammation.

ACPA induces pain-like behaviour

Intravenous administration of human polyclonal ACPA (anti-CCP2 IgG purified from RA sera, Paper I) evoked mechanical hypersensitivity in mice. The reduction in the tactile threshold was observed already at day three, and lasted up to 28 days post-injection. ACPA-injected mice also presented heightened thermal hypersensitivity (both cold and heat) in comparison to animals injected with either CCP2 flow through (FT) or saline. Spontaneous pain like-behaviour (non-evoked) was evaluated by monitoring the animals total movement, directional walking and rearing. Only ACPA-injected mice presented a significant reduction in the three parameters addressed. No signs of inflammation or arthritis were detected, suggesting that the pain-like behaviour (evoked and spontaneous) is a result of ACPA pro-nociceptive effect and not due to classic inflammation-related molecules. The same experiments were performed with two other ACPA pools, prepared from different RA patient samples, in different mouse strains and similar results were obtained, which indicates that ACPA-mediated pain is independent of the autoantibodies batch and mouse strain. Murinised monoclonal ACPA obtained from synovial fluid B cells from patients with RA evoked similar pain like-behaviour as that observed with human polyclonal ACPA. This finding rules out the possibility of the induction of pain being the result of an immune response against human antibodies.

One of the ACPA pools was utilized to investigate if mice developed arthritis in another set of experiments. After a single intravenous injection of human purified autoantibodies followed (three hours later) by an intraperitoneal injection of lipopolysaccharide no arthritis was registered (data not shown). Mice injected with a cocktail of anti-collagen antibodies developed transient arthritis. Additional experiments need to be performed to further explore whether or not ACPA possess arthritogenic potential.

The location of antibodies in mice organs was evaluated seven days post-injection. ACPA were specifically found in joint, bone marrow, skin, DRG, adipose tissue and spleen whereas RA non-CCP2 reactive IgG and IgG purified from healthy donors presented a similar distribution, yet broader. These observations suggested that ACPA may have preferential organ targets, which may give clues on the pro-nociceptive mechanism of action. Furthermore, these findings bring out the potential to identify novel non-described ACPA targets.

ACPA-pain mediated mechanisms

The direct effect of ACPA in primary peripheral neurons was investigated as a possible ACPA-pain mediated mechanism. Primary cultures of mouse DRG were stimulated with ACPA or FT (at 1 µg/ml) followed by KCl (50 mM, positive control) stimulation at the end of the experiment. The intracellular concentration of Ca^{2+} was measured in order to assess cells that were able to depolarize (thus, capable of generating an action potential). From the total number of cells utilized in the assay 80% was activated by KCl and from those only 2.5% and 1.7% were activated in response to ACPA and FT, respectively. Additionally, ACPA did not induce inward currents in small nociceptive neurons. These observations lead us to conclude that ACPA does not induce Ca^{2+} flux or inward current, which indicates lack of direct modulation of neuronal excitability.

ACPA-injected mice did not present the classical signs of inflammation. Histological evaluations showed no cell infiltration or hyperplasia in the synovia. In addition, mRNA analysis showed no significant differences between levels of certain chemokines (CXCL5, CCL2), cytokines (TNF, Il-1β and IL6), matrix metalloproteinases, inflammatory enzymes (Cox2), and mast cell proteases in ankle joint lysates from ACPA-injected mice in comparison to saline –administered animals. Interestingly, mRNA levels of chemokines CXCL1 (human IL-8 analogue) and CXCL2 were found elevated in the ankle joint of ACPA-injected mice. No difference was observed in the mRNA levels of both CXCL1 and CXCL2 in FT and saline-injected mice.

Immunohistochemistry analysis revealed that ACPA specifically binds non-permeabilised $CD68^{+}$ cells which, due to location and morphology, appear to be osteoclasts. Thereafter, mouse osteoclasts were treated with ACPA or FT and the release of human IL-8 analogs (CXCL1/2) was examined. A significant increase in the levels of CXCL1 was found in supernatants from ACPA-treated mouse osteoclasts whereas no difference was observed in supernatants from FT or saline-treated osteoclasts. Immunostainings also showed that, in the bone marrow, ACPA binds $CD68^{+}$cells located near the calcitonin gene related peptide (CGRP)-positive sensory fibers, known to transmit pain/nociceptive sensation.

In order to investigate whether ACPA induces pain-like behaviour via CXCL1/2 mechanism, additional *in vivo* experiments were performed. Previous studies demonstrated that CXCL chemokines can induce pain-like behaviour (385-388). In study II, the injection of either CXCL1/2 alone or CXCL1 and CXCL2 together evoked a rapid mechanical hypersensitivity (significant decrease of tactile threshold within two hours post-injection that lasted at least 24 hours). Mice injected with the monoclonal ACPA D10 and B2 were treated with CXCR1/2 antagonist (reparixin) for six consecutive days. ACPA-induced mechanical and thermal hypersensitivity was partially reversed by the treatment with reparixin. Control antibody and reparixin itself did not evoke hypersensitivity.

Taken together, our data suggest that ACPA induces a pain-like behaviour through binding to $CD68^+$ osteoclasts and leading to secretion of CXCL1, which in turn activates neighboring CXCR2 expressing sensory nerves. This mechanism provides evidence for the role of ACPA mediating pain in those seropositive patients that although successfully treated for inflammation still report pain. In addition, the presence of certain circulating ACPA before clinical onset may explain the joint pain in these individuals (384, 389, 390). Additionally, it appears that distinct polyclonal and monoclonal ACPA induce pain differently. One possible explanation is the different fine specificities ACPA encompass. In Paper IV we describe the purification and characterization of a sub-group of citrullinated-fibrinogen reactive ACPA that will in the future be utilized to address whether certain ACPA fine specificities (e.g. fibrinogen) are indeed responsible for clinical phenotypes such as non-inflammatory mediated pain in RA.

3. Targeting of human ACPA using fibrinogen-derived peptides (Paper III and IV)

We have previously identified endogenously citrullinated fibrinogen peptides in synovial tissue from patients with RA (372): within the α chain - Arg573Cit (563-583) and Arg591Cit (580-600), referred to as Cit573 and Cit591; within the β chain - Arg72Cit (62-81), and Arg74Cit (62-81), referred to as Cit72 and Cit74 (Table 8 methods section).

Endogenously citrullinated fibrinogen peptides constitute autoantigens in RA (Paper III)

Both the citrullinated and non-modified version of these peptides were placed onto a chemically modified glass slide and ACPA response was evaluated by incubating the peptides with sera from RA patients (EIRA cohort). These peptide microarray experiments revealed that ~50% of the patients sera (n=927) tested were positive for at least one of the four peptides whereas <2% of healthy controls presented a weak response (the diagnostic specificity for anti-CCP2 was 98.4%). Within the CCP2-positive population, 65% and 15% of the patients were positive for the peptides Cit573 and Cit59, respectively; 35% and 53% of the patients were positive for Cit72 and Cit74, respectively. Within the anti-CCP2 negative RA group, Cit72 and Cit591 held the highest reactivity with a percentage of 8.4% and 6.1%, respectively; Cit573and Cit74 displayed lower reactivity, 5.5% and 4.4%, respectively. These observations are in line with recent studies showing reactivity against citrullinated peptides in the CCP-negative RA population (391, 392). Previously, these peptides were tested in a pre-RA cohort (180). Notably, increased titres of anti-Cit591 and anti-Cit72 antibodies were observed earlier in the disease course, which suggests a possible involvement in the clinical

onset. On the other hand, anti-Cit573 and anti-Cit74 autoantibodies were elevated at/after the clinical onset, suggesting instead a possible part in disease progression.

Anti-CCP2 IgG are inhibited by fibrinogen-derived peptides (Paper III and IV)

The capacity of Cit573, 591, 72 and 74 to block human ACPA was evaluated in a set of competition assays (**Paper III**). After incubating different pools of affinity purified human anti-CCP2 IgGs (ACPA or aCCP2 IgG pool I and II) with increasing amounts of each citrullinated peptide (or non-modified control), the remaining anti-CCP2 reactivity was measured and the blocking capacity estimated.

Cit573 was the peptide that presented the best blocking capacity by inhibiting up to 84% of aCCP2 IgG (pool I) present in the assay. **Cit591** followed displaying a blocking percentage of 63%. Peptides from the β chain (**Cit72** and **Cit74**) exhibited lower aCCP2 IgG inhibition in comparison to Cit573 and Cit591, 35 and 26%, respectively. The IC_{50} for each peptide was estimated in order to provide additional information on the inhibition capacity. Concordant with the best dose-response curve, Cit573 registered the best $IC_{50,}$ 59 μM, compared to Cit591 which was calculated as 194 μM.
The enhanced aCCP2 IgG blocking capacity after mixing Cit573 and Cit591 (91%) suggests that the pool of aCCP2 IgG is also composed of non-cross-reactive antibodies, albeit the high blockade percentage exerted by the single peptides. This observation extends our findings in paper I where we described that anti-CCP2 IgG purified from distinct patients bind differently to CCP2 and other ACPA fine specificities. The same experiment was performed utilizing autoantibodies purified from a second pool of RA plasma as well as from a pool of eleven SF samples. Fifty per cent of aCCP2 IgG pool II was inhibited by both Cit573 and Cit591 with an IC_{50} of 548 μM and 412 μM, respectively. In paper III only results regarding aCCP2 IgG pool I and II were presented, however, the inhibition percentage displayed by the peptides when incubated with the antibodies purified from SF mimicked that observed for aCCP2 IgG pool II.

Linear truncated and cyclic versions of the peptide Cit573 (also named [Cit573]fib(563-583), Table 9 in methods) were designed in order to assess whether these new structures improve ACPA blocking capacity. Notably, the cyclic version of Cit573 (**Cit573Cyc, c[Cit573]fib(563-583)**) blocked ~90% of all antibodies present in the assay regardless the pool tested (IC_{50} of 28 and 71 μM for aCCP2 IgG pool I and II, respectively). Amino acids located on both extremities of the linear peptide appear not to influence the binding capacity of the truncated peptides (Cit573Lin1, [Cit573]fib(566-580) and Lin2, [Cit573]fib(567-577) in comparison to the full length peptide ([Cit573]fib(563-583)). Truncated **[Cit573]fib(566-580)** peptide blocked 75% of aCCP2 IgG pool I (IC_{50} 51 μM) and 47% of aCCP2 IgG pool II (IC_{50} 186 μM); Truncated **[Cit573]fib(567-577)** peptide blocked 69% and 40% of aCCP2 IgG pool I and II, respectively (IC_{50} 123 and 105 μM).

The concept of neutralizing circulating autoantibodies has been explored in an animal model of autoimmune cardiomyopathy whereby anti-β1-adrenergic receptor (β1AR) antibodies were shown to mediate myocardial damage. A cyclic peptide (COR-1) that mimics the main epitope of β1AR neutralized anti-β1AR antibodies and prevented myocardial injury, and furthermore reverted dysfunction of the heart when administered to rats once a month in the

course of 3 to 6 months (366, 393, 394). Besides peptides, other small molecules such as aptamers have been tested in animal models of SLE and shown to neutralize anti-dsDNA and thereby prevent autoantibody-mediated renal injury (395). Likewise, aptamers were also demonstrated to neutralize anti-β1AR antibodies purified from patients suffering from cardiomyopathies (396).

Development of a high binding ACPA inhibitor – SFTI-1 scaffold opportunity (Paper IV)

In paper IV we further developed the conception of an ACPA blocker. The purpose of such a compound would be to block ACPA in patients, preventing chronic pain and other debilitating symptoms of RA (365). Figure 16 (under conclusions and future perspectives section) illustrates the strategy. We designed a new ACPA blocker which specifically targets anti-Cit573 autoantibodies (**Paper IV**). This new molecule comprises the truncated version [Cit573]fib(566-580) (Table 8 and 9, Figure 14) linked to the sunflower trypsin inhibitor-1 (SFTI-1). In addition to the required specificity of this blocking compound (termed **s1[Cit573]fib(566-580)**), the stability and non-cytotoxicity are major concerns that we overcame by using the cyclic plant-derived SFTI-1. The circular peptide backbones and the presence of disulfide bond(s) provide these scavenger great stability. In fact, the ability SFTI-1 holds to accommodate bioactive sequences within its three-dimensional framework, and simultaneously keep the stability, has been demonstrated (397, 398). SFTI-1 appears to be safe, with no immunogenic or cytotoxic properties reported (399, 400).

As described in paper III, [Cit573]fib(563-583) linear truncated versions [Cit573]fib(566-580) and [Cit573]fib(567-577) blocked ~70% of aCCP2 IgG from pool I and <50% of aCCP2 IgG pool II (**Paper IV**). **Cyclic (c) [Cit573]fib(563-583)** reach up to 90% aCCP2 IgG inhibition of both pools but when [Cit573]fib(566-580) was head-to-tail cyclized (**c[Cit573]fib(566-580)**) the blocking capacity marginally decreased (86% and 72% inhibition of aCCP2 IgG pool I and II, respectively). Interestingly, the arginine (**c[Arg573]fib(566-580**)) and an alanine (**c[Ala573]fib(566-580**)) control versions displayed up to 50% aCCP2 IgG inhibition. The amino acid residues flanking the citrulline residue have a high impact in the binding affinity to the autoantibodies possibly because of the adopted unstructured conformation, as it was observed in the NMR studies. Although mainly random, the amino acids that flank citrulline in several known ACPA epitopes (e.g. within CEP-1, Cit-vim and Cit-fib) appear to be preferentially small and rather neutral (401).

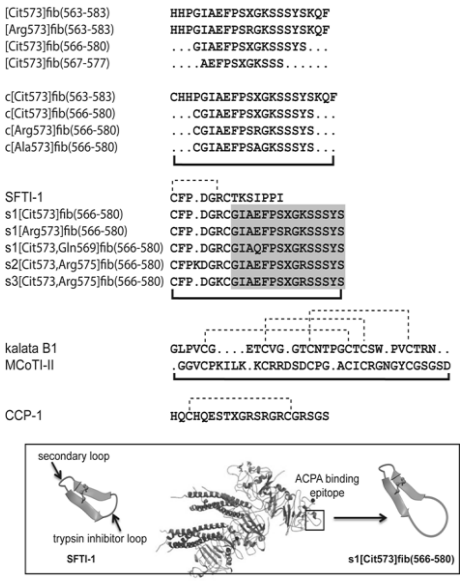


Figure 14 Sequences of the grafted and native peptides and a schematic of the ACPA inhibitor design strategy.

S1[Cit573]fib(566-580) acquired improved stability both in whole blood and serum in comparison to the linear and cyclic counterpart versions. Above 90% of s1[Cit573]fib(566-580) was retained in blood after five hours whereas the linear peptides were degraded after one hour. In the competition assay, s1[Cit573]fib(566-580) was able to block 79% of

aCCP2 IgG pool I with an IC_{50} of 20 μM. A lower percentage of antibodies was blocked when testing aCCP2 IgG pool II (61%, IC_{50} of 87 μM) as it was shown for all the other peptides.

Optimizing s1[Cit573]fib(566-580) for ACPA purification and improved affinity (Paper IV)

In order to facilitate the binding of peptides to affinity chromatography columns and ELISA plates the lysine mutant **s2[Cit573,Arg575]fib(566-580)** was designed. S2[Cit573,Arg575]fib(566-580) was coupled to a NHS-activated affinity column and utilized to purify Cit573-reactive autoantibodies. Approximately 0.33% of total IgG from RA patients (average concentration of 32 μg/mL) was found to react against [Cit573]fib(563-583). These autoantibodies were used in experiments that enabled a more accurate measurement of the blocking capacity and binding affinity of scaffold peptides.
In s2[Cit573,Arg575]fib(566-580) the insertion of a lysine in the secondary loop of SFTI-1 disrupted the β hairpin conformation which led to a decreased stability in solution in comparison to the original s1 scaffold. S2[Cit573,Arg575]fib(566-580) rapidly degraded to < 80% after three hours of incubation in both blood and serum. Therefore, a new mutant, s3[Cit573,Arg575]fib(566-580), was designed in which the arginine of the structural loop was substituted by a lysine. This peptide showed structural similarities to s1[Cit573]fib(566-580) and as a consequence improved stability in comparison to mutant s2. Forty nine per cent of s3[Cit573,Arg575]fib(566-580) remained after three hours incubation in blood.

The blocking capacity of the new mutants was explored in the competition assay in which both the aCCP2 IgG pool I and aCit573 IgG were tested. S3[Cit573,Arg575]fib(566-580) was the mutant with the best performance since 40 nmol of the compound blocked 88% aCCP2 IgG. The isolation of ACPA subtypes (aCit573 IgG) and their use in the competition assay demonstrated an improvement in the blocking efficiency of s3[Cit573,Arg575]fib(566-580 (97% inhibition) in comparison to aCCP2 IgG blockade. S2[Cit573,Arg575]fib(566-580) blocked 44% and 66% of aCCP2 IgG pool I and aCit573 IgG, respectively. Binding affinity assays (fluorescence correlation spectroscopy) demonstrated and confirmed that the mutant s3[Cit573,Arg575]fib(566-580) binds aCit573 IgG with high affinity (K_d=2 nM). K_d for aCCP2 IgG was registered 17 nM.

In summary, paper III and IV provide proof-of-principle that affinity purified ACPA and specific ACPA fine specificities (aCit573 IgG) can be neutralized *in vitro* by stable molecules structurally based on endogenously citrullinated fibrinogen peptides. Anti-Cit573 IgG bind scaffolded fibrinogen peptides with high affinity. Furthermore, we propose that such molecules may be applied for development of potent ACPA blocking compounds.

4. Characterization of extracellular histidyl-tRNA synthetase in myositis (Paper V)

Histidyl-tRNA synthetase (HisRS or Jo1) is one of the most prevalent autoantigens in myositis. Autoantibodies that target this protein (anti(a)-Jo1/aHisRS) are present in a large percentage of patients with myositis as well as in patients with anti-synthetase syndrome, characteristic clinical phenotype that involves organs other than the muscle (e.g. the lung) (276, 285, 293).

HisRS is present in circulation – Lung and muscle as possible sources of extracellular HisRS

In paper V we described the presence of HisRS in extracellular compartments such as serum, plasma and BAL. Patients with myositis presented elevated levels of HisRS in sera in comparison to HC and RA patients. Furthermore, patients with aJo1+ myositis appear to have higher amount of HisRS in circulation in comparison to aJo1- population.
Extracellular HisRS was also detected in BAL fluid. Interestingly, a strong signal dictating the presence of HisRS was found in HC (and sarcoidosis patients) contrary to what was observed for healthy sera in which very little protein was detected. This observation led us to speculate whether HisRS, known to be a cytoplasmic enzyme, could additionally be present in extracellular compartments in physiological conditions. Furthermore, we questioned if HisRS detected in circulation could be originated and released from the lung under unique circumstances such as chronic inflammation, as observed in myositis. One possible source within the lung could be the alveolar epithelium known to express HisRS in the native and granzyme B proteolytic sensitive conformation (this conformation is recognized by sera from aJo1+ patients and thus, may be autoantigenic) (328). Pneumocytes, alveolar macrophages and endothelial cells in the bronchus have also been shown to express HisRS (402). Additional evidences suggest a connection between HisRS and the lung in an autoimmune context: 1) association between aJo1 and ILD (403, 404); 2) aJo1 associates with HLA DRB1*03 genetic background and smoking (57); 3) patients with HLA DRB1*03 genotype display selective T cell-receptor V-gene usage in BAL and muscle (333).

A possible additional source of systemic HisRS in myositis could be the muscle. Previous studies strikingly demonstrated an increase of HisRS expression on muscle fibers undergoing regeneration, rather than in fully differentiate myotubes (98). In fact, HisRS protein levels in healthy muscle are very low, compared to muscle from myositis patients (98, 402). As it was recently described that recombinant HisRS and a HisRS splice variant (lacking the entire catalytic domain but still recognized by aJo1+ sera) are secreted from mouse C2C12 myoblasts (310) we speculated whether regenerating muscle fibers were the origin of extracellular HisRS found in the sera of patients with myositis. The WB analysis of muscle homogenates from several different myositis patients revealed significant expression of HisRS. Additionally, myoblasts and low-grade differentiating myotubes derived from a dermatomyositis patient also showed HisRS protein expression. However, no HisRS could be detected in the supernatants collected from myoblasts treated with TNF-α. Additional experiments are required in order to further dissect the muscle as the origin of exogenous HisRS.

The presence of a cytoplasmic protein such as HisRS in extracellular comportments is also supported by a growing body of evidence demonstrating the secretion of several aaRS by different types of cells. Tyrosyl-RS and threonyl-RS, both autoantigens in myositis, were described to be secreted by endothelial cells (405, 406). Threonyl-RS was in fact shown to be secreted by ovarian cancer cells and to be present in sera of cancer patients (407). Glycyl-RS, another autoantigenic aaRS described in myositis, was detected in healthy sera and it was found to be released by macrophages (408).

Extracellular HisRS and the 'X-factor'

The presence of HisRS extracellularly was verified by performing pre-adsorption experiments. BAL fluid and sera were placed onto nitrocellulose membranes and incubated in parallel with either anti-HisRS-N-terminal antibody or with the anti-HisRS-N-terminal antibody previously bound to an excess of recombinant HisRS. Although we could confirm that HisRS was indeed the protein detected in BAL fluid and sera, we came across an unexpected finding (Figure 15a, right panel).

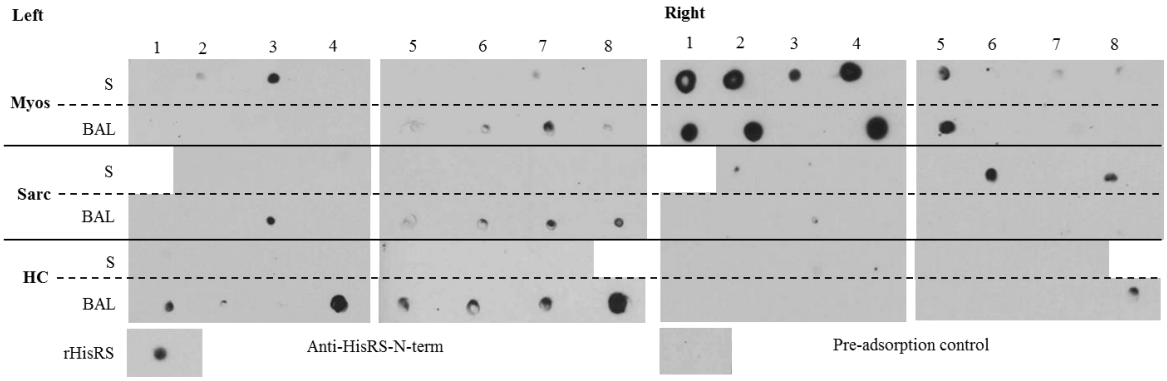


Figure 15a HisRS is present in circulation and in BAL fluid. The pre-adsorption control experiments reveal the presence of a 'HisRS enhancing binding factor' translated by the intense black dot mainly observed in BAL fluid and sera from myositis patients. Numbers 1 to 8 represent different individuals. Myos, myositis; S, sarcoidosis; HC, healthy controls; BAL, bronchoalveolar lavage fluid.

In order to address the nature of the 'enhancing binding factor' we first sought for the existence of IC comprising HisRS and IgG linked to C1q, but no C1qIC could be detected in BAL from either aJo1+ or aJo1- myositis BAL. Noteworthy, IgG-depleted BAL presented a similar black signal compared to that observed in the original BAL suggesting that the presence of anti-Jo1 IgG autoantibodies may not solely contribute to the enhancing binding factor (Figure 15b).

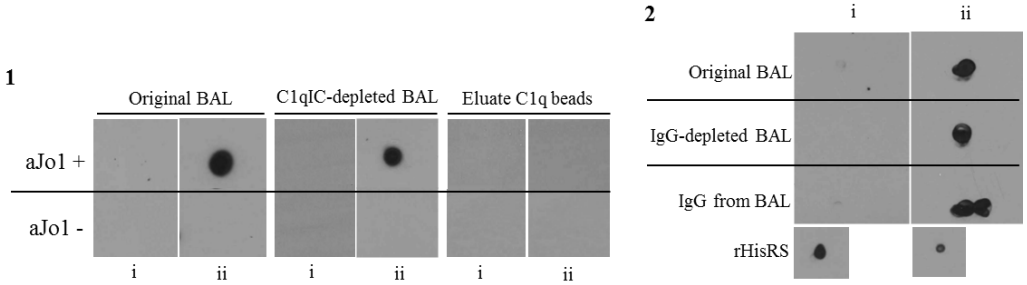


Figure 15b Possible origins of HisRS enhancing binding factor. i, normal protocol; ii, pre-adsorption protocol.

Subsequently, we investigated the presence of anti-Jo1 isotypes other than IgG. Both aJo1 IgA and aJo1 IgM could be simultaneously detected in sera and BAL fluid from at least three of the eight myositis patients analysed. The presence of autoantibodies in BAL fluid has been previously described. ACPA were revealed to be enriched in the lungs of RA patients (83). Furthermore, early ACPA+ RA individuals (with no lung disease) were recently found to contain germinal centers, B cells and plasma cells in the lung (84). Our observations of anti-Jo1 autoantibodies in BAL fluid from myositis patients are in line with these studies.

The BAL fluid was subjected to additional experiments in order to address the presence of autoantibodies other than aJo1 and therefore, find alternative explanations for the nature of the binding factor. Notably, among the different antibody reactivities tested (anti- Ro60/SSA, La/SSB, Sm, Sm RNP, U1 RNP, Scl-70, dsDNA, CENPB, RIBO, pm-scl, and PCNA) only antibodies targeting TRIM21 (Ro52) could be detected in BAL from myositis patients. Moreover, a positive correlation between the presence of aTRIM21 IgG and aJo1 IgG in BAL was found (p= 0,007; Spearman ρ^2= 0,881). A summary of the findings concerning HisRS and BAL fluid from myositis patients is listed in Table 10. Of note, the three patients positive for the anti-Jo1 isotypes also contain aTRIM21 IgG, ILD, the binding factor and absence of extracellular HisRS. All patients positive for aJo1 IgG were diagnosed with ILD.

Table 10 Information on diagnosis of ILD and presence of anti-Jo1 isotypes, anti-TRIM21 IgG, extracellular HisRS and 'enhancing binding factor' in BAL from patients with myositis.

Myositis patient	ILD*	aJo1 IgG	aJo1 IgA	aJo1 IgM	aTRIM21 IgG	HisRS	Binding factor
1	+	+	+	+	+	-	+
2	+	+	+	+	+	-	+
3	-	-	-	-	-	+	-
4	+	+	+	+	+	-	+
5	+	+	-	-	-	+	+
6	-	-	-	-	-	-	-
7	+	+	-	-	-	+	-
8	-	-	-	-	+	+	-

* ILD, Interstitial lung disease; + positive/presence; – negative/absence.

Although at this point we have no definitive proof for the genesis of the enhancing binding factor, it is tempting to speculate whether a molecular interaction between HisRS and TRIM21 may be the root cause. Both these two molecules have been suggested to be targets of a coupled immune response and to co-occur in myositis (409). In addition, patients with ASS and with aTRIM21 IgG were shown to have worse ILD in comparison to those patients that did not present aTRIM21 IgG (410). Additional experiments are required in order to confirm the origin of HisRS enhancing binding factor.

Extracellular HisRS and a novel non-canonical function

Platelets are key mediators of the immune response and inflammation. For instance, platelets, known to express TLR4 and 2, were shown to trap bacteria in liver sinusoids and pulmonary capillaries by inducing NETS via TLR4 (411). Interestingly, HisRS has been attributed a role in triggering innate immunity in myositis through TLR4 and 2 (via common MyD88 signaling cascade) (331, 332). Doubled knockout mice for TLR4 and 2 presented reduced inflammation that had been induced by HisRS immunization (332). Moreover, the generation of class-switched autoantibodies in a murine model of myositis induced by intramuscular injection of recombinant HisRS was dependent on TLR4 (412). Raynaud's phenomenon, common clinical feature of ASS, was found to be associated with platelet-derived microparticles and further, with enhanced platelet activation and aggregation (413-415). In line, patients with DM present high titers of platelet-derived microparticles (416). As a result of detecting HisRS exogenously, we sought for non-canonical functions namely, the effect of HisRS on platelets and discovered that HisRS activates platelets (by increasing CD62P

expression) in a dose-response manner at low picomolar concentrations. The discovery that another aaRS (tryrosyl-RS) acts as a thrombopoietic agent supports our findings and collectively suggests that aaRS may display important platelet-related functions.

Anti-Jo1 autoantibodies

Recombinant human HisRS was employed to prepare an affinity column for purification of autoantibodies targeting HisRS from human sera (aJo1). The methodology utilized follows the scheme published in Paper I (Figure 13, results and discussion section). Anti-Jo1 IgG were estimated to constitute 1.5% of the total myositis IgG pool, existing in an average concentration of 180 µg/ml in myositis sera from aJo1+ patients. This values fall in the range of anti-CCP2 IgG reactivity in RA patients, as we similarly demonstrated in Paper I.

In conclusion, paper V provides further evidence for a role of HisRS in myositis with lung involvement, which may be due to: 1) the presence of the protein that once in extracellular compartments such as serum and BAL fluid may be more amenable to undergo PTMs; 2) a coupled immune response between HisRS with TRIM21. Furthermore, we suggest that when exogenously HisRS may perform additional non-canonical functions such as platelets activation.

CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis was devoted to the study of two chronic autoimmune diseases. RA and myositis are complex, multifactorial conditions that express heterogeneous clinical presentations. In both cases, patients can be grouped in seropositive or seronegative according to the existence of certain circulating autoantibodies. Particularly, antibodies targeting citrullinated proteins/peptides (ACPA) are characteristic of a major population of patients with RA displaying more erosive disease; anti-Jo1 (HisRS) autoantibodies are typically associated with myositis and together with seven other so far described, aaRS, further classify a sub-group of patients with ASS. The studies performed in this thesis have contributed to a better understanding of the auto-antibody/antigen related molecular mechanisms underlying RA and myositis.

The development of a methodology to purify autoantibodies (Paper I) opened a full new array of possibilities to study the autoimmune response engaged by ACPA and anti-Jo1 IgG in patients with RA and myositis, respectively. This method was employed throughout my PhD, providing me with autoantibodies of different reactivities which enabled the studies presented in the five papers that constitute this thesis. Purified ACPA anti-CCP2 IgG) have been additionally utilized to explore their pathogenic role in bone destruction (379), synovial fibroblasts migration (417), identification of novel targets (418) and glycosylation patterns (219). In **Paper I** we demonstrated that CCP2 acts as a surrogate antigen for a variety of ACPA fine specificities and we showed that those ACPAs bind to *in vivo* and *in vitro* generated citrullinated proteins. The methodology developed herein, provided an accurate measurement of ACPA concentration and proportion in plasma and SF of RA patients.

It would be interesting to address the correlation between individual ACPA levels (and also fine specificities) and the treatment the patients underwent at time of sample retrieval. Such an assessment may offer insights into which therapies can successfully be applied to ACPA+ patients. It would also be interesting to investigate new possible ACPA targets in the synovial tissue. In **Paper I** we demonstrated ACPA binding to synovial tissue and SF cells. On-going two-dimensional gel electrophoresis and mass spectrometry studies may soon provide additional clues on novel ACPA autoantigens (372).

The evidence that purified human ACPA induces pain independent of inflammation (**Paper II**) provides explanatory clues to the molecular mechanisms underlying the inflammation and transmission of pain in RA. Furthermore, the occurrence of ACPA before signs of arthritis could explain the joint pain that sometimes precedes clinical onset. Interestingly, we discovered that ACPA does not directly activate primary sensory neurons, but rather seems to induce pain through binding to $CD68^+$ osteoclasts in the joints, leading to release of CXCL1 and consequently activation of nearby sensory neurons.

Purification of the different known ACPA fine specificities and injection in mice may help to understand which populations of ACPA are responsible for inducing pain-like behaviour. Although no concrete proof is available, certain ACPA reactivities seem to occur earlier than others (anti-Cit591 and anti-Cit-vim 60–75) (180). Other ACPA reactivities would also be of value for testing in the *in vivo* setting (anti-Cit573, anti-CEP-1 or anti-CitC1). It would be very interesting to investigate whether ACPA induced pain-like behaviour could be reverted

or prevented by using specific blockers. Such strategy was the founding idea of Paper III and IV.

The development of new blocking molecules capable of neutralizing pathogenic ACPA introduces a new therapeutic approach intended to specifically target ACPA+RA patients. The concept of personalized therapies has been increasingly suggested due to the possibility to treat patients more specifically (that is, targeting molecular mechanisms known to be responsible for the disease presentation in a specific group of individuals) and with less side-effects.

Paper III demonstrates the autoantigenic potential of four citrullinated fibrinogen peptides, previously shown to be endogenously citrullinated (372). Those antigens were tested for the capacity to block purified human ACPA (aCCP2 IgG), and [Cit573]fib(563-583) peptide revealed to be a good candidate. In order to improve ACPA specific binding and blood stability, a shorter linear version of [Cit573]fib(563-583) was inserted into a scavenger (SFTI-1, **Paper IV**). The new molecule, s1[Cit573]fib(566-580), proved to be more stable than both the linear counterpart and the cyclic versions of [Cit573]fib(563-583). In addition, the arginine version, s1[Arg573]fib(566-580), did not display significant aCCP2 IgG binding. The development of an additional mutant, s2[Cit573,Arg575]fib(566-580), provided a new tool for the successful purification of anti-Cit573 autoantibodies from a mixture of total RA IgG. These autoantibodies will be injected in mice and the pain-like behaviour will be assessed, as performed for total aCCP2 IgG in Paper II. Additionally, the mutant s3[Cit573]fib(566-580) will be tested for the capacity to reduce and/or prevent pain in aCCP2 IgG -injected mice (as well as for anti-Cit573 IgG, in case this reactivity would induce pain). Figure 16 illustrates the treatment strategy we suggest based on the findings from Paper III and IV.

Autoantigens provide crucial clues for the understanding of the molecular mechanisms that govern autoimmune diseases such as RA and myositis. In this thesis special emphasis was given to HisRS, the major autoantigen known for myositis. In **Paper V** we demonstrated the presence of HisRS in extracellular components such as serum, plasma and BAL fluid. Patients with myositis presented higher levels of circulating HisRS in comparison to HC and RA patients. Interestingly, BAL fluid from HC and sarcoidosis patients presented significant amounts of extracellular HisRS.

HisRS is a cytoplasmic enzyme involved in the protein synthesis. The presence of this enzyme outside the cell in physiological conditions is indicative of additional novel non-canonical functions, similar to what has been described for other synthetases (311). It has been demonstrated that proteins such as aaRS underwent evolutionary pressure over the phylogenetic tree. aaRS from higher eukaryotic organisms were appended additional domains not found in prokaryotes (311). For example, the WHEP domain of HisRS is known to contain the main epitope target of anti-Jo1 autoantibodies (313, 321) and is not present in bacteria and in lower eukaryotes. This domain was demonstrated to be essential for HisRS chemoattractant properties (99) and it contains a granzyme B cleavage site that originates a proteolytic structure known to be highly expressed in the lung (328).

We herein show that HisRS activates platelets in a dose-response manner. The mechanism of action is so far unknown. One possibility may be through activation of TLR receptors since it

is acknowledged that platelets express those receptors and HisRS was shown to engage in innate immunity processes via TLR2 and 4 (331, 332).

BAL fluid from patients with myositis revealed the presence of an unknown 'enhancing binding factor'. In an attempt to understand the meaning and origin of such factor we discovered that BAL fluid from patients with myositis contain anti-Jo1 IgG, IgA and IgM. In addition, anti-TRIM21 IgG were also found in BAL (and sera) from the same patients and were further shown to be positively associated with anti-Jo1 IgG. No C1qIC could be detected in the BAL fluid, ruling out the possibility of IC, comprising C1q, aJo1 and HisRS, as the enhancing factor. These findings support a previous study in which the authors raised a possible coupled immune response involving both molecules, HisRS and TRIM21 (409). Although these findings do not directly explain the origin of the binding factor, they nevertheless provide additional evidence for the role of HisRS (and to some extent TRIM21) in mediating the development of autoimmunity in patients with myositis and lung involvement. Figure 17 illustrates one current hypothesis for the initiation and development of myositis autoimmunity.

We speculated whether circulating HisRS originates from the lungs (as it was found in BAL fluid). However, other possible sources should be taking into consideration. One possibility may well be the regenerating muscle, known to express elevated amounts of autoantigenic HisRS in myositis (98). Our data do not support the muscle as the point of exogenous HisRS origin. However, additional studies are required to fully answer this question. One interesting approach to investigate the cellular origin of exogenous HisRS would be to determine whether circulating HisRS is bound to microparticles. If so, the origin of those microparticles may help us discover from which cells HisRS is been released. *In vitro* grown myotubes may be utilized in order to investigate if HisRS is released from the cells in response to different types of stimuli (e.g. other cytokines), not herein investigated.

The development of a quantitative method (e.g. ELISA) to determine exact circulating HisRS values is of major importance. Preliminary data (not shown) suggest that HisRS values in serum from patients with myositis may vary overtime. To assess a possible correlation between auto- antibody/antigen levels and whether there is as an association between those and the therapies administered to the patients may not only give insight into the immune mechanism behind aJo1+ myositis but it may also be used as a future biomarker.

In paper V anti-Jo1 autoantibodies were purified and briefly characterized. Similarly to what we have found for ACPA, aJo1 constitute ~1.5% of the total IgG pool in sera from patients with myositis. These autoantibodies are currently being tested in cell cultures of myoblasts and myotubes with the purpose to characterize possible pathogenic effects (e.g. induction of cell death, release of proinflammatory cytokines and modulation of muscle contractility machinery).

In conclusion, this thesis has contributed to the knowledge underlying RA and IIM immunological mechanisms and provided the first steps for the development of an autoantibody-targeted therapy.

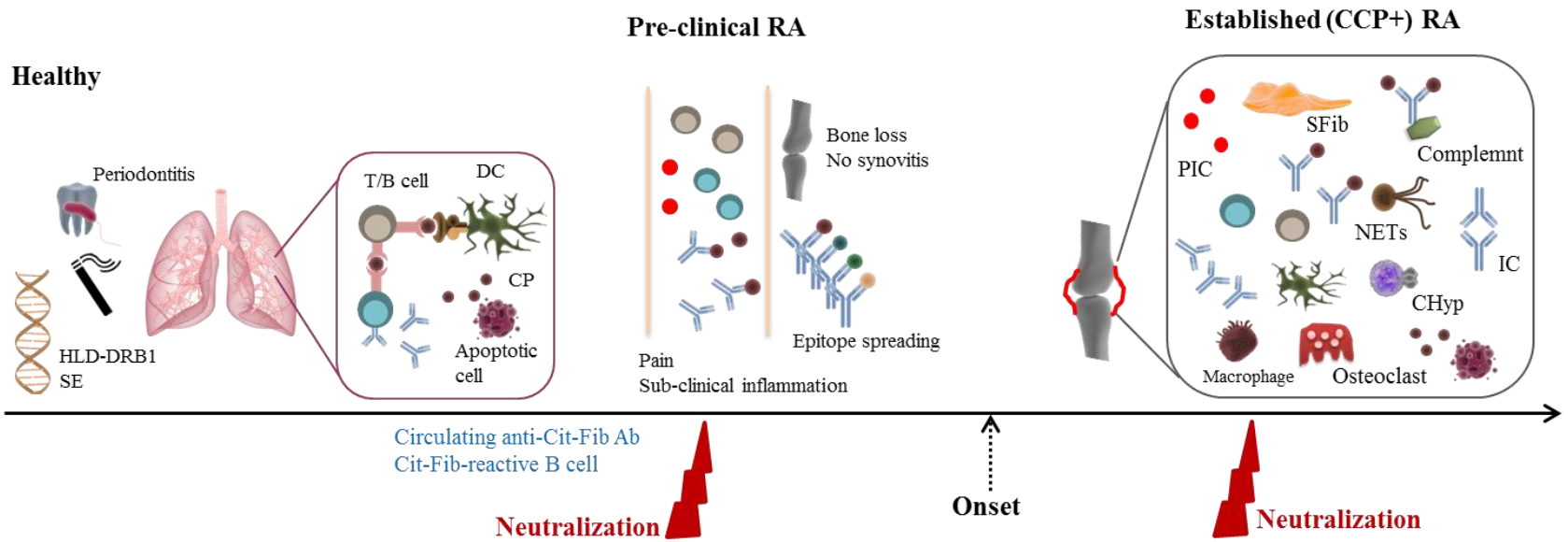


Figure 16 In the hypothetical model presented for CCP+ RA, we propose the neutralization of citrullinated-fibrinogen reactive autoantibodies (Anti-Cit-Fib Ab) and B cells (Cit-Fib-reactive B cell) as a complementary strategy to treat this subset of patients.

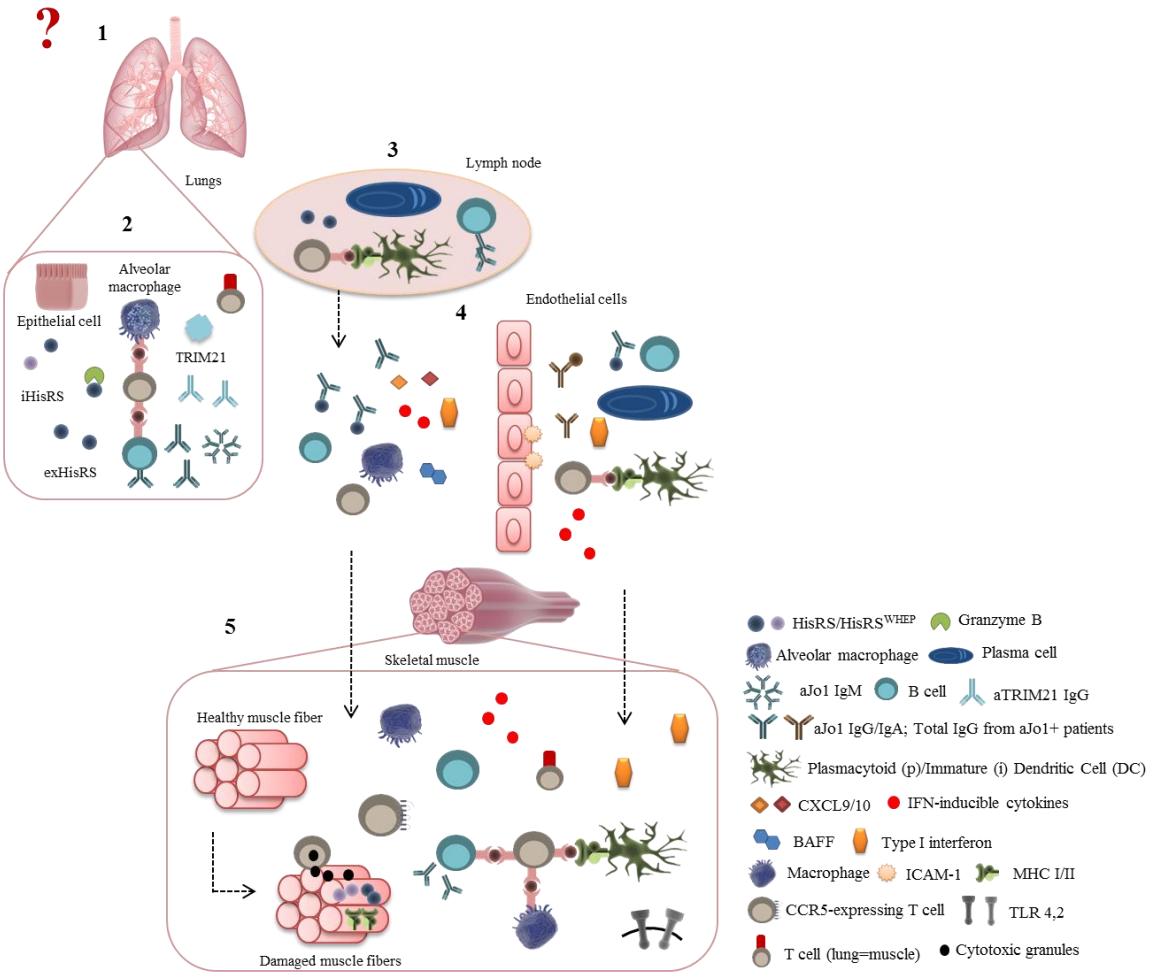


Figure 17 Based on the findings described in this thesis we added new pieces of evidence to the hypothesis of lungs as the site of autoimmune origin in myositis. HisRS is physiologically present in extracellular compartments (intracellular (i) HisRS expressed in lung epithelial cells; extracellular (ex) HisRS present in BAL for instance). In adverse conditions (e.g. inflammation caused by smoking or viral infection) HisRS may undergo post-translational modifications leading to a tolerance breach and consequent local production of autoantibodies of various isotypes.

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